

FORM PTO-1390 (REV 11-2000)	U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE	ATTORNEY'S DOCKET NUMBER 35-213
TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371		U.S. APPLICATION NO. (if known, see 37 C.F.R. 1.5) 09/913159 Unknown
INTERNATIONAL APPLICATION NO. PCT/EP99/09759	INTERNATIONAL FILING DATE 3 December 1999	PRIORITY DATE CLAIMED 12 February 1999

TITLE OF INVENTION

VIRAL VACCINE

APPLICANT(S) FOR DO/EO/US

SCHREIBER

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. ☒ This is an express request to begin national examination procedures (35 U.S.C. 371(f)). The submission must include items (5), (6), (9) and (21) indicated below.
4. ☒ The U.S. has been elected by the expiration of 19 months from the priority date (Article 31).
5. A copy of the International Application as filed (35 U.S.C. 371(c)(2)).
 - a. ☐ is attached hereto (required only if not communicated by the International Bureau).
 - b. ☒ has been communicated by the International Bureau.
 - c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US).
6. ☒ An English language translation of the International Application as filed (35 U.S.C. 371(c)(2)).
 - a. ☒ is attached hereto.
 - b. ☐ has been previously submitted under 35 U.S.C. 154(d)(4).
7. ☒ Amendments to the claims of the International Application under PCT Article 34
 - a. ☐ are attached hereto (required only if not communicated by the International Bureau).
 - b. ☒ have been communicated by the International Bureau.
 - c. ☐ have not been made; however, the time limit for making such amendments has **NOT** expired.
 - d. ☐ have not been made and will not be made.
8. ☐ An English language translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
9. ☐ An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).
10. ☒ An English language translation of the annexes of the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

Items 11 To 20 below concern document(s) or information included:

11. ☐ An Information Disclosure Statement under 37 C.F.R. 1.97 and 1.98.
12. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 C.F.R. 3.28 and 3.31 is included.
13. ☒ A **FIRST** preliminary amendment.
14. ☐ A **SECOND** or **SUBSEQUENT** preliminary amendment.
15. ☐ A substitute specification.
16. ☐ A change of power of attorney and/or address letter.
17. ☒ A computer-readable form of the sequence listing in accordance with PCT Rule 13ter.2 and 35 U.S.C. 1.821-1.825.
18. ☐ A second copy of the published international application under 35 U.S.C. 154(d)(4).
19. ☐ A second copy of the English language translation of the international application under 35 U.S.C. 154(d)(4).
20. ☒ Other items or information. PTO-1449/ International Search Report/ Sequence Listing/ Front page of the PCT Publication/ Certification of Translation/Letter re: Sequence Listing

CT/PTO 10 AUG 2001

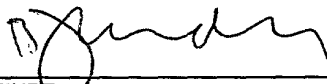
U.S. APPLICATION NO. 09/913159		ATTORNEY'S DOCKET NUMBER 35-213	
21. <input checked="" type="checkbox"/> The following fees are submitted:		CALCULATIONS PTO USE ONLY	
BASIC NATIONAL FEE (37 C.F.R. 1.492(a)(1)-(5)): -- Neither international preliminary examination fee (37 C.F.R. 1.482) nor international search fee (37 C.F.R. 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO\$1000.00 -- International preliminary examination fee (37 C.F.R. 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO.....\$860.00 -- International preliminary examination fee (37 C.F.R. 1.482) not paid to USPTO but international search fee (37 C.F.R. 1.445(a)(2)) paid to USPTO\$710.00 -- International preliminary examination fee (37 C.F.R. 1.482) paid to USPTO but all claims did not satisfy provisions of PCT Article 33(1)-(4).....\$690.00 -- International preliminary examination fee (37 C.F.R. 1.482) paid to USPTO and all claims satisfied provisions of PCT Article 33(1)-(4).....\$100.00			
ENTER APPROPRIATE BASIC FEE AMOUNT =			
Surcharge of \$130.00 for furnishing the oath or declaration later than <input type="checkbox"/> 20 <input checked="" type="checkbox"/> 30 months from the earliest claimed priority date (37 C.F.R. 1.492(e)).		\$ 130.00	
CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE
Total Claims	54	-20 = 34	X \$18.00
Independent Claims	13	-3 = 10	X \$80.00
MULTIPLE DEPENDENT CLAIMS(S) (if applicable)			\$270.00
TOTAL OF ABOVE CALCULATIONS =			\$ 2402.00
<input type="checkbox"/> Applicant claims small entity status. See 37 CFR 1.27. The fees indicated above are reduced by 1/2.			0.00
SUBTOTAL =			\$ 2402.00
Processing fee of \$130.00, for furnishing the English Translation later than <input type="checkbox"/> 20 <input type="checkbox"/> 30 months from the earliest claimed priority date (37 C.F.R. 1.492(f)).			0.00
TOTAL NATIONAL FEE =			\$ 2402.00
Fee for recording the enclosed assignment (37 C.F.R. 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 C.F.R. 3.28, 3.31). \$40.00 per property			0.00
Fee for Petition to Revive Unintentionally Abandoned Application (\$1240.00 - Small Entity = \$620.00)			0.00
TOTAL FEES ENCLOSED =			\$ 2402.00
			Amount to be:
			refunded \$
			Charged \$

- a. ☒ A check in the amount of \$2402.00 to cover the above fees is enclosed.
- b. ☐ Please charge my Deposit Account No. 14-1140 in the amount of \$_____ to cover the above fees. A duplicate copy of this form is enclosed.
- c. ☒ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 14-1140. A duplicate copy of this form is enclosed.
- d. ☒ The entire content of the foreign application(s), referred to in this application is/are hereby incorporated by reference in this application.

NOTE: Where an appropriate time limit under 37 C.F.R. 1.494 or 1.495 has not been met, a petition to revive (37 C.F.R. 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:

NIXON & VANDERHYE P.C.
1100 North Glebe Road, 8th Floor
Arlington, Virginia 22201-4714
Telephone: (703) 816-4000


SIGNATURE

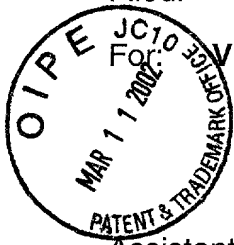
B. J. Sadoff
NAME

36,663 **August 10, 2001**
REGISTRATION NUMBER Date

#7

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of

SCHREIBERAtty. Ref.: **35-213**Serial No. **09/913,159**Group: **Unknown**Filed: **August 10, 2001**Examiner: **Unknown****VIRAL VACCINE**

* * * * *

March 11, 2002Assistant Commissioner for Patents
Washington, DC 20231

Sir:

AMENDMENT

Responsive to the Notification dated January 18, 2002, and further to the Request of February 6, 2002, entry and consideration of the following amendments and remarks are requested.

IN THE SPECIFICATION

Amend the specification as follows:

Insert the attached Sequence Listing in place of the copy of the same filed August 10, 2001.

REMARKS

Reconsideration is requested.

T00160-051660

A copy of the Notification of Missing Requirements dated January 18, 2002, and Raw Sequence Listing Error Report attached to the same, are attached. The specification has been amended to include the attached Sequence Listing.

The attached paper and computer-readable copies of the Sequence Listing are the same. No new matter has been added. A separate Statement to this effect is attached.

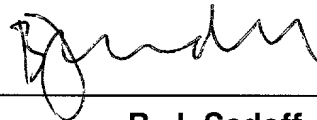
Nothing further is believed to be required in response to the Notification of Missing Requirements dated January 18, 2002, however the Office is requested to contact the undersigned if otherwise.

An early and favorable Action on the merits is requested.

Respectfully submitted,

NIXON & VANDERHYE P.C.

By: _____



B. J. Sadoff

Reg. No. 36,663

BJS:eaw

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Arlington, VA 22201-4714
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Facsimile: (703) 816-4100

#7

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of

SCHREIBERAtty. Ref.: **35-213**Serial No. **09/913,159**Group: **Unknown**Filed: **August 10, 2001**Examiner: **Unknown****VIRAL VACCINE**

* * * * *

March 11, 2002Assistant Commissioner for Patents
Washington, DC 20231

Sir:

STATEMENT

The attached paper and computer-readable copies of the Sequence Listing are the same. No new matter has been added.

Respectfully submitted,

NIXON & VANDERHYE P.C.By: **B. J. Sadoff**Reg. No. **36,663****BJS:eaw**1100 North Glebe Road, 8th Floor
Arlington, VA 22201-4714
Telephone: (703) 816-4000
Facsimile: (703) 816-4100

RECEIVED MAR 11 2002



PCT09

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DATE: 04/29/2002
TIME: 11:20:41

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Use of n and/or Xaa have been detected in the Sequence Listing. Please review the Sequence Listing to ensure that a corresponding explanation is presented in the <220> to <223> fields of each sequence which presents at least one n or Xaa.

Seq#:4; N Pos. 99

FOOTNOTES: 62 FEB 66

VERIFICATION SUMMARY

DATE: 04/29/2002

PATENT APPLICATION: US/09/913,159A

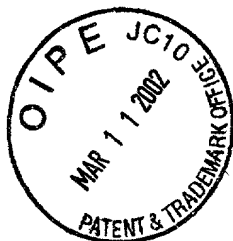
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FOOTNOTES



11 MAR 2002

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	370					375					380					
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385					390					395					400	
Glu	Gly	Ser	Asn	Asn	Thr	Glu	Gly	Ser	Asp	Thr	Ile	Thr	Leu	Pro	Cys	
			405						410					415		
Arg	Ile	Lys	Gln	Phe	Ile	Asn	Met	Trp	Gln	Glu	Val	Gly	Lys	Ala	Met	
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Thr Lys Ala Lys Arg Arg Val Val Gln Arg Glu Lys Arg Ala Val Gly 500 505 510		
Ile Gly Ala Leu Phe Leu Gly Phe Leu Gly Ala Ala Gly Ser Thr Met 515 520 525		
Gly Cys Thr Ser Met Thr Leu Thr Val Gln Ala Arg Gln Leu Leu Ser 530 535 540		
Asp Ile Val Gln Gln Gln Asn Asn Leu Leu Arg Ala Ile Glu Ala Gln 545 550 555 560		
Gln His Leu Leu Gln Leu Thr Val Trp Gly Ile Lys Gln Leu Gln Ala 565 570 575		
Arg Ile Leu Ala Val Glu Arg Tyr Leu Lys Asp Gln Gln Leu Leu Gly 580 585 590		
Ile Trp Gly Cys Ser Gly Lys Leu Ile Cys Thr Thr Ala Val Pro Trp 595 600 605		
Asn Ala Ser Trp Ser Asn Lys Ser Leu Glu Gln Ile Trp Asn Asn Met 610 615 620		
Thr Trp Met Glu Trp Asp Arg Glu Ile Asn Asn Tyr Thr Ser Leu Ile 625 630 635 640		
His Ser Leu Ile Glu Glu Ser Gln Asn Gln Gln Glu Lys Asn Glu Gln 645 650 655		
Glu Leu Leu Glu Leu Asp Lys Trp Ala Ser Leu Trp Asn Trp Phe Asn 660 665 670		
Ile Thr Asn Trp Leu Trp Tyr Ile Lys Leu Phe Ile Met Ile Val Gly 675 680 685		
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Arg Val Arg Gln Gly Tyr Ser Pro Leu Ser Phe Gln Thr His Leu Pro 705 710 715 720		
Ile Pro Arg Gly Pro Asp Arg Pro Glu Gly Ile Glu Glu Glu Gly Gly 725 730 735		
Glu Arg Asp Arg Asp Arg Ser Ile Arg Leu Val Asn Gly Ser Leu Ala 740 745 750		

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755 760 765

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835 840 845

Leu Glu Arg Ile Leu Leu
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<210> 3

<211> 107

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of the artificial sequence:
oligonucleotide for cloning

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<210> 4

<211> 120

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of the artificial sequence:
oligonucleotide for cloning

<220>

<221> misc_feature

<222> (97)..(99)

<223> Sequence at this position: (GA)(AT)(GATC), ie.
base at position 97 can be G or A, base at
position 98 can be A or T, and base at
position 99 can be G, A, T or C.

<400> 4

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FOOTNOTES: 051660

100T80-EST-60

<210> 5
<211> 17
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of the artificial sequence:
sequencing primer

<400> 5
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17

<210> 6
<211> 17
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of the artificial sequence:
sequencing primer

<400> 6
aaaactgtgc gttacaa

17

<210> 7
<211> 17
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of the artificial sequence:
sequencing primer

<400> 7
gtaaaacgac ggccagt

17

<210> 8
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<212> DNA
<213> Artificial Sequence

<220>
<223> Description of the artificial sequence:
sequencing primer

<400> 8
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17

<210> 9
<211> 2148

<212> DNA
<213> Artificial Sequence

<220>
<223> Description of the artificial sequence: synthetic DNA

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<222> (3)..(9)
<223> BstEII cleavage site

<220>
<221> misc_feature
<222> (2143)..(2148)
<223> BamHI cleavage site

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<211> 6229

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<220>

<223> Description of the artificial sequence: PI-932
gene cassette, comprising the cleavage sites for
restriction enzymes BspTl, PstI, BclI, EcoRI,
BglIII, PvuII, XbaII, NheI

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100750" 537660

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of

SCHREIBER

Atty. Ref.: 35-213

Serial No. Unknown

Group:

U.S. National Phase of PCT/EP99/09759

Filed: August 10, 2001

Examiner:

For: VIRAL VACCINE

* * * * *

August 10, 2001

Assistant Commissioner for Patents
Washington, DC 20231

Sir:

LETTER

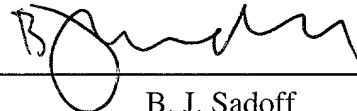
The attached paper and computer readable copies of the Sequence Listing are the same.

No new matter has been added.

Respectfully submitted,

NIXON & VANDERHYE P.C.

By: _____



B. J. Sadoff
Reg. No. 36,663

1100 North Glebe Road, 8th Floor
Arlington, VA 22201-4714
Telephone: (703) 816-4000
Facsimile: (703) 816-4100

SEQUENCE LISTING

<110> Strathmann AG & Co.

<120> Virus-Vaccine

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<141>

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<213> Human immunodeficiency virus

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<220>
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Thr Thr Thr Leu Phe Cys Ala Ser Asp Ala Lys Ala Tyr Asp Thr Glu
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Val His Asn Val Trp Ala Thr His Ala Cys Val Pro Thr Asp Pro Asn
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Pro Gln Glu Val Val Leu Val Asn Val Thr Glu Asn Phe Asn Met Trp
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Lys Asn Asp Met Val Glu Gln Met His Glu Asp Ile Ile Ser Leu Trp
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Asp Gln Ser Leu Lys Pro Cys Val Lys Leu Thr Pro Leu Cys Val Ser
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Leu Lys Cys Thr Asp Leu Lys Asn Asp Thr Asn Thr Asn Ser Ser Ser
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Tyr Lys Leu Asp Ile Val Pro Ile Asp Asn Thr Ser Tyr Arg Leu Ile
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Ser Cys Asn Thr Ser Val Ile Thr Gln Ala Cys Pro Lys Val Ser Phe
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Glu Pro Ile Pro Ile His Tyr Cys Ala Pro Ala Gly Phe Ala Ile Leu
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Lys Cys Asn Asn Lys Thr Phe Asn Gly Thr Gly Pro Cys Thr Asn Val
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Leu Leu Leu Asn Gly Ser Leu Ala Glu Glu Asp Val Val Ile Arg Ser
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Ala Asn Phe Thr Asp Asn Ala Lys Thr Ile Ile Val Gln Leu Asn Thr
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FOOTNOTES: 651-660

Ser Val Glu Ile Asn Cys Thr Arg Pro Asn Asn Asn Thr Arg Lys Ser
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Arg Ile Lys Gln Phe Ile Asn Met Trp Gln Glu Val Gly Lys Ala Met
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Tyr Ala Pro Pro Ile Ser Gly Gln Ile Arg Cys Ser Ser Asn Ile Thr
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FOUO 667650

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oligonucleotide for cloning

<220>
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<222> (97)..(99)
<223> Sequence at this position: (GA)(AT)(GATC), ie.
base at position 97 can be G or A, base at
position 98 can be A or T, and base at
position 99 can be G, A, T or C.

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<220>
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<400> 5
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<210> 6
<211> 17
<212> DNA
<213> Artificial Sequence

<220>
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sequencing primer

<400> 6
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<210> 7
<211> 17
<212> DNA
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<223> Description of the artificial sequence:
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000133-08101
000133-08101

<210> 8
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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of

SCHREIBER

Atty. Ref.: 35-213

Serial No. Unknown

Group:

U.S. National Phase of PCT/EP99/09759

Filed: August 10, 2001

Examiner:

For: VIRAL VACCINE

* * * * *

August 10, 2001

Assistant Commissioner for Patents
Washington, DC 20231

Sir:

PRELIMINARY AMENDMENT

In order to place the above-identified application in better condition for examination, please amend the application as follows:

IN THE CLAIMS

Please substitute the following amended claims for corresponding claims previously presented. A copy of the amended claims showing current revisions is attached.

3. (Amended) Protein vaccine according to claim 1 , characterized in that it comprises a mixture of GP120 proteins of HIV which in each case differ from each other in their amino acid sequence in the region of the V2 loop and/or of the V3 loop.

6. (Amended) DNA vaccine according to claim 4 , characterized in that the mixture contains $\geq 10^3$ and preferably $\geq 10^4$ DNA molecules which differ from each other in their nucleic acid sequence.

7. (Amended) DNA vaccine according to claim 4, characterized in that it codes for a mixture of structurally different GP120 proteins of HIV, in which the vaccine contains a mixture of DNA molecules, the nucleic acid sequences of which differ from each other in the region coding for the V2 loop and/or in the region coding for the V3 loop.

11. (Amended) Nucleic acid sequence according to claim 9 , characterized in that the sequence is modified by the introduction of silent mutations.

12. (Amended) Nucleic acid sequence according to claim 9, characterized in that it contains the sequence given in SEQ ID NO: 9.

17. (Amended) Nucleic acid sequence according to claim 15 or a single-stranded nucleic acid sequence which contains the region coding for the V2 loop and/or the region of GP120 coding for the V3 loop, characterized in that in the V3 loop a 247 bp-long Bg1II-XbaI fragment or a 283 bp-long Bg1II-NheI fragment is exchanged for a modified fragment, and in the V2 loop a 139 bp-long PstI-Bc1I fragment or a 339 bp-long PstI-EcoRI fragment is exchanged for a modified fragment, the fragment/the fragments in each case containing inosine, a nucleic acid exchange or a mutation at at least 6 positions, the nucleic acid sequence being complementary to a nucleic acid sequence characterized in that it contains the sequence given in SEQ ID NO: 12 , characterized in that the fragment/the fragments contain(s) inosine, a nucleic acid exchange or a mutation at 9 to 20 positions.

18. (Amended) Double-stranded DNA which comprises hybrids of the single-stranded nucleic acid sequence according to claim 15 with a single-stranded nucleic

acid sequence which contains the region coding for the V2 loop and/or the region of GP120 coding for the V3 loop, characterized in that in the V3 loop a 247 bp-long Bg1II-XbaI fragment or a 283 bp-long Bg1II-NheI fragment is exchanged for a modified fragment, and in the V2 loop a 139 bp-long PstI-Bc1I fragment or a 339 bp-long PstI-EcoRI fragment is exchanged for a modified fragment, the fragment/the fragments in each case containing inosine, a nucleic acid exchange or a mutation at at least 6 positions, the nucleic acid sequence being complementary to a nucleic acid sequence characterized in that it contains the sequence given in SEQ ID NO: 12.

27. (Amended) Expression vector, characterized in that it contains an inserted nucleic acid sequence according to claim 9.

33. (Amended) Vector mixture according to claim 30, characterized in that the plasmids can be expressed in *E. coli* as host cell.

34. (Amended) Vector mixture according to claim 30, characterized in that the plasmids can be expressed in eukaryotic cells, preferably in Cos, CHO or BHK cells, as host cells.

40. (Amended) Process according to claim 38 , characterized in that the nucleic acid sequence coding for a viral protein is the sequence according SEQ ID NO: 1 or SEQ ID NO: 11 or a fragment of same.

41. (Amended) Process for the preparation of the vector mixture according to claim 33, characterized in that plasmids, the nucleic acid sequences of which in each case differ from each other in the region coding for the V2 loop and/or in the region coding for the V3 loop in each case through random distribution of the bases at the

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varied nucleotide positions, are ligated into a vector which can be expressed in host cells.

43. (Amended) Process for the preparation of the host cells composing transforming *E.coli*. with a vector mixture according to claim 30 .

44. (Amended) Process for the preparation of a protein vaccine which comprises a mixture of viral protein molecules, characterized in that the molecules are sequence variants of a single viral protein or of part of same, the mixture containing $\geq 10^2$ sequence variants, which is obtainable by expression of a plasmid-DNA mixture which, because of the variation of nucleotide positions, contains randomly distributed sequence combinations , said process comprising cultivating host cells according to claim 35 under conditions which allow the expression of the mixture of viral protein sequence variants.

45. (Amended) Process for the preparation of a DNA vaccine which codes for a mixture of structurally different virus proteins, characterized in that the vaccine contains a mixture of sequence variants of a viral DNA molecule or of part of same, the mixture containing $\geq 10^2$ DNA molecules which differ from each other in their nucleic acid sequence, where the mixture, because of the variation of nucleotide positions, contains randomly distributed sequence combinations wherein said process is carried out according to claim 41 , wherein the plasmids are ligated into a vector which can be expressed in host cells of the organism to be vaccinated.

46. (Amended) A method of preparing a vaccine comprising forming a mixture of structurally different viral proteins which are sequence variants of a viral protein or of part of same, for the prevention and/or therapy of a virus infection in humans.

47. (Amended) A method of preparing a vaccine comprising forming a mixture according to claim 23 for the prevention and/or therapy of a HIV infection in humans.

48. (Amended) A method of preparing a vaccine comprising forming a mixture of DNA molecules which code for sequence variants of a viral protein or of part of same, for the prevention and/or therapy of a virus infection in humans.

49. (Amended) A method of preparing a vaccine comprising forming a nucleic acid mixture according to claim 19 for the prevention and/or therapy of a virus infection in humans.

50. (Amended) A method of preparing a vaccine comprising forming the nucleic acid mixture according to claim 19 for the expression in host cells selected from the group consisting of *E. coli*, Cos, CHO and BHK cells.

51. (Amended) A method of producing a protein mixture which comprises sequence variants of the GP120 protein, characterized in that it is a mixture of proteins which contain amino acid sequences which in each case differ from each other in the V2 loop and/or in the V3 loop, the mixture being obtainable by expression of a plasmid DNA mixture which, because of the variation of nucleotide positions, contains randomly distributed sequence combinations; said method comprising expressing a vector mixture which contains a mixture of plasmids which contains an inserted double-stranded DNA which comprises hybrids of the single-stranded nucleic acid sequence according to claim 15 with a single-stranded nucleic acid sequence which contains the region coding for the V2 loop and/or the region of GP120 coding for the V3 loop, characterized in that in the V3 loop a 247 bp-long Bg1II-XbaI fragment or a 283 bp-long Bg1II-NheI fragment is exchanged for a modified fragment, and in the V2 loop a 139 bp-long PstI-Bc1I fragment or a 339 bp-long PstI-EcoRI fragment is exchanged for a modified fragment, the fragment/the fragments in each case containing inosine, a nucleic acid exchange or a mutation at at least 6 positions, the nucleic acid sequence being complementary to a nucleic acid sequence characterized in that it contains the sequence given in SEQ ID NO: 12; characterized in that the nucleic acid sequences of the plasmids differ in each case from each other in the region coding for the V2 loop and/or in the region coding for the V3 loop, where the mixture of the plasmids, because

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of the variation of nucleotide positions, contains randomly distributed sequence combinations .

52. (Amended) A method of preparing a protein mixture which comprises sequence variants of the GP120 protein, characterized in that it is a mixture of proteins which contain amino acid sequences which in each case differ from each other in the V2 loop and/or in the V3 loop, the mixture being obtainable by expression of a plasmid DNA mixture which, because of the variation of nucleotide positions, contains randomly distributed sequence combinations, said method comprising culturing a host cell according to claim 35 .

54. (Amended) Pharmaceutical composition for the prevention and/or therapy of a virus infection, characterized in that it comprises a protein mixture and a nucleic acid mixture, the protein mixture comprising sequence variants of a viral protein or of part of same, and the nucleic acid mixture comprising DNA molecules which code for sequence variants of a viral protein or of part of same, said composition comprising a protein mixture according to claim 23 and a nucleic acid mixture which comprises double-stranded DNAs, the nucleic acid sequences of which are derived from the *env* sequence in SEQ ID NO: 1 or SEQ ID NO: 11 or a fragment of same, characterized in that the nucleic acid sequences in each case differ from each other in the region coding for the V2 loop and/or in the region coding for the V3 loop .

REMARKS

The above amendments are made to place the claims in a more traditional format. Attached hereto is a marked-up version of the changes made to the claims by the current amendment. The attached pages are captioned "**Version With Markings To Show Changes Made.**"

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Respectfully submitted,

NIXON & VANDERHYE P.C.

By: _____



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VERSION WITH MARKINGS TO SHOW CHANGES MADE

IN THE CLAIMS

3. (Amended) Protein vaccine according to claim 1 [or 2], characterized in that it comprises a mixture of GP120 proteins of HIV which in each case differ from each other in their amino acid sequence in the region of the V2 loop and/or of the V3 loop.

6. (Amended) DNA vaccine according to claim 4 [or 5], characterized in that the mixture contains $\geq 10^3$ and preferably $\geq 10^4$ DNA molecules which differ from each other in their nucleic acid sequence.

7. (Amended) DNA vaccine according to [one of claims 4 to 6] claim 4, characterized in that it codes for a mixture of structurally different GP120 proteins of HIV, in which the vaccine contains a mixture of DNA molecules, the nucleic acid sequences of which differ from each other in the region coding for the V2 loop and/or in the region coding for the V3 loop.

11. (Amended) Nucleic acid sequence according to claim 9 [or 10], characterized in that the sequence is modified by the introduction of silent mutations.

12. (Amended) Nucleic acid sequence according to [claims 9 to 11] claim 9, characterized in that it contains the sequence given in SEQ ID NO: 9.

17. (Amended) Nucleic acid sequence according to claim 15 or [16] a single-stranded nucleic acid sequence which contains the region coding for the V2 loop and/or the region of GP120 coding for the V3 loop, characterized in that in the V3 loop a 247 bp-long Bg1II-XbaI fragment or a 283 bp-long Bg1II-NheI fragment is exchanged for a modified fragment, and in the V2 loop a 139 bp-long PstI-Bc1I fragment or a 339 bp-

long PstI-EcoRI fragment is exchanged for a modified fragment, the fragment/the fragments in each case containing inosine, a nucleic acid exchange or a mutation at at least 6 positions, the nucleic acid sequence being complementary to a nucleic acid sequence characterized in that it contains the sequence given in SEQ ID NO: 12, characterized in that the fragment/the fragments contain(s) inosine, a nucleic acid exchange or a mutation at 9 to 20 positions.

18. (Amended) Double-stranded DNA which comprises hybrids of the single-stranded nucleic acid sequence according to claim 15 [or 17] with [the] a single-stranded nucleic acid sequence [according to claim 16 or 17] which contains the region coding for the V2 loop and/or the region of GP120 coding for the V3 loop, characterized in that in the V3 loop a 247 bp-long Bg1II-XbaI fragment or a 283 bp-long Bg1II-NheI fragment is exchanged for a modified fragment, and in the V2 loop a 139 bp-long PstI-Bc1I fragment or a 339 bp-long PstI-EcoRI fragment is exchanged for a modified fragment, the fragment/the fragments in each case containing inosine, a nucleic acid exchange or a mutation at at least 6 positions, the nucleic acid sequence being complementary to a nucleic acid sequence characterized in that it contains the sequence given in SEQ ID NO: 12.

27. (Amended) Expression vector, characterized in that it contains an inserted nucleic acid sequence according to [claims 9 to 14]claim 9.

33. (Amended) Vector mixture according to [one of claims 30 to 32]claim 30, characterized in that the plasmids can be expressed in *E. coli* as host cell.

34. (Amended) Vector mixture according to [one of claims 30 to 32]claim 30, characterized in that the plasmids can be expressed in eukaryotic cells, preferably in Cos, CHO or BHK cells, as host cells.

40. (Amended) Process according to claim 38 [or 39], characterized in that the nucleic acid sequence coding for a viral protein is the sequence according SEQ ID NO: 1 or SEQ ID NO: 11 or a fragment of same.

41. (Amended) Process for the preparation of the vector mixture according to [claims 33 and 34]claim 33, characterized in that plasmids, the nucleic acid sequences of which in each case differ from each other in the region coding for the V2 loop and/or in the region coding for the V3 loop in each case through random distribution of the bases at the varied nucleotide positions, are ligated into a vector which can be expressed in host cells.

43. (Amended) Process for the preparation of the host cells [according to claim 35 or 36, characterized in that the host cells are transformed] composing transforming *E.coli*, with a vector mixture according to [claims] claim 30 [to 32].

44. (Amended) Process for the preparation of a protein vaccine which comprises a mixture of viral protein molecules, characterized in that the molecules are sequence variants of a single viral protein or of part of same, the mixture containing $\geq 10^2$ sequence variants, which is obtainable by expression of a plasmid-DNA mixture which, because of the variation of nucleotide positions, contains randomly distributed sequence combinations [according to one of claims 1 to 3, characterized in that the host cells are cultivated] , said process comprising cultivating host cells according to [one of claims 35 to 37] claim 35 under conditions which allow the expression of the mixture of viral protein sequence variants.

45. (Amended) Process for the preparation of a DNA vaccine which codes for a mixture of structurally different virus proteins, characterized in that the vaccine contains a mixture of sequence variants of a viral DNA molecule or of part of same, the mixture containing $\geq 10^2$ DNA molecules which differ from each other in their nucleic acid sequence, where the mixture, because of the variation of nucleotide positions, contains

randomly distributed sequence combinations wherein said [according to [one of claims 4 to 8, characterized in that the] process is carried out according to claim 41 [or 42], wherein the plasmids [according to the invention being] are ligated into a vector which can be expressed in host cells of the organism to be vaccinated.

46. (Amended) A method of preparing a vaccine comprising forming a [Use of a] mixture of structurally different viral proteins which are sequence variants of a viral protein or of part of same, for [the preparation of a vaccine for] the prevention and/or therapy of a virus infection in humans.

47. (Amended) A method of preparing a vaccine comprising forming a mixture [Use of a protein mixture] according to [one of claims 23 to 25]claim 23 [for the preparation of a vaccine] for the prevention and/or therapy of a HIV infection in humans.

48. (Amended) A method of preparing a vaccine comprising forming [Use of] a mixture of DNA molecules which code for sequence variants of a viral protein or of part of same, for [the preparation of a vaccine for] the prevention and/or therapy of a virus infection in humans.

49. (Amended) A method of preparing a vaccine comprising [Use of] forming a nucleic acid mixture according to [claims 19 to 22]claim 19 for the [preparation of a vaccine for the] prevention and/or therapy of a virus infection in humans.

50. (Amended) A method of preparing a vaccine comprising [Use of] forming the nucleic acid mixture according to [one of claims 19 to 22]claim 19 for the [preparation of a vector mixture according to one of claims 30 to 32 which can be expressed] expression in host cells[, the host cells being] selected from the group consisting of *E. coli*, Cos, CHO and BHK cells.

51. (Amended) A method of producing a protein mixture which comprises sequence variants of the GP120 protein, characterized in that it is a mixture of proteins

which contain amino acid sequences which in each case differ from each other in the V2 loop and/or in the V3 loop, the mixture being obtainable by expression of a plasmid DNA mixture which, because of the variation of nucleotide positions, contains randomly distributed sequence combinations; said method comprising [Use of the vector mixture according to one of claims 30 to 32] expressing a vector mixture which contains a mixture of plasmids which contains an inserted double-stranded DNA which comprises hybrids of the single-stranded nucleic acid sequence according to claim 15 with a single-stranded nucleic acid sequence which contains the region coding for the V2 loop and/or the region of GP120 coding for the V3 loop, characterized in that in the V3 loop a 247 bp-long Bg1II-XbaI fragment or a 283 bp-long Bg1II-NheI fragment is exchanged for a modified fragment, and in the V2 loop a 139 bp-long PstI-Bc1I fragment or a 339 bp-long PstI-EcoRI fragment is exchanged for a modified fragment, the fragment/the fragments in each case containing inosine, a nucleic acid exchange or a mutation at at least 6 positions, the nucleic acid sequence being complementary to a nucleic acid sequence characterized in that it contains the sequence given in SEQ ID NO: 12; characterized in that the nucleic acid sequences of the plasmids differ in each case from each other in the region coding for the V2 loop and/or in the region coding for the V3 loop, where the mixture of the plasmids, because of the variation of nucleotide positions, contains randomly distributed sequence combinations [for the expression of a protein mixture according to one of claims 23 to 25].

52. (Amended) A method of preparing a protein mixture which comprises sequence variants of the GP120 protein, characterized in that it is a mixture of proteins which contain amino acid sequences which in each case differ from each other in the V2 loop and/or in the V3 loop, the mixture being obtainable by expression of a plasmid DNA mixture which, because of the variation of nucleotide positions, contains randomly distributed sequence combinations, said method comprising culturing a [Use of the] host cell according to [one of claims 35 to 37]claim 35 [for the preparation of a protein mixture according to one of claims 23 to 25].

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54. (Amended) Pharmaceutical composition for the prevention and/or therapy of a virus infection, characterized in that it comprises a protein mixture and a nucleic acid mixture, the protein mixture comprising sequence variants of a viral protein or of part of same, and the nucleic acid mixture comprising DNA molecules which code for sequence variants of a viral protein or of part of same, said composition comprising [according to claim 53, characterized in that it comprises] a protein mixture according to claim 23 [claims 23 to 25] and a nucleic acid mixture which comprises double-stranded DNAs, the nucleic acid sequences of which are derived from the *env* sequence in SEQ ID NO: 1 or SEQ ID NO: 11 or a fragment of same, characterized in that the nucleic acid sequences in each case differ from each other in the region coding for the V2 loop and/or in the region coding for the V3 loop [according to claims 19 to 22].

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VIRUS VACCINE

The present invention relates to a pharmaceutical composition or a vaccine which comprises a mixture of viral protein molecules which are sequence variants of a single viral protein or of part of same. The invention furthermore relates inter alia to a DNA vaccine which codes for a mixture of structurally different virus proteins, the vaccine containing a mixture of sequence variants of a viral DNA molecule or of part of same, which code for sequence variants of a viral protein or part. According to a preferred version of the invention, the viral proteins are sequence variants of the GP120 protein of the human immunodeficiency virus (HIV) which differ from each other in their amino acid sequence in the region of the V2 loop and/or of the V3 loop, preferably both the V2 and the V3 loop. The invention furthermore relates to the preparation of the virus vaccines including the intermediate stages or constructs, preparation processes and uses connected with them.

In many viral infections, in particular in the HIV-1 infection, a strong immune defence is observed, which is in

In the prior art, for the treatment of viral illnesses, such as e.g. the polio virus (Horaud F et al., Biologicals, 1993, 21:311-316), Hanta virus (Ulrich R et al., 1998 Vaccine 16:272-280; Schmaljohn CS et al., 1992 vaccine 10:10-13), Lassa virus (Morrison HG et al., 1989 Virology 171:179-188; Clegg JC et al., 1987 Lancet 2:186-188), hepatitis A virus (Clemens et al., 1995 J. Infect. Dis. 171:Suppl.1:p.44-p.49; Andre et al., 1990 Prog. Med. Virol. 37:72-95) and hepatitis B virus infection (McAleer et al., 1984 Nature 307:178-180) but also the HIV infection (Egan et al., 1995 J. Infect. Dis. 171:1623-1627, Kovac et al., 1993 J. Clin. Invest. 92:919-928), only single, genetically unmodified, specific virus antigens or single inactivated virus strains have been used for studies of suitable vaccination strategies. In the case of HIV, for example, the external envelope proteins of two virus strains were up until now prepared for vaccine experiments on humans (MN and SF2) (Zolla-Pazner et al., J. Infect. Dis. 178 (1998) 1502-1506). Both GP120 molecules differ from each other in the amino acid sequence, but particularly in the amino acid

sequence of the variable regions, such as e.g. of the V3 loop. Because of the different V3 loop sequences, the two virus strains have different phenotypic properties. The HIV-1 MN strain is a virus variant which preferentially infects macrophages and cells which possess the viral co-receptor CCR5. Viruses of the SF2 type, on the other hand, multiply preferentially in T cells and use the viral co-receptor CXCR4. Such viruses are therefore also called T cell trophs (e.g. HIV strain) or macrophagotrophs (e.g. HIV strain MN). Vaccination experiments have been carried out in chimpanzees with these two GP120 variants. It has been shown in these experiments that an immune response can be induced which not only protects against the two HIV strains MN and SF2, but is also capable of preventing infection by other virus strains, but also by HIV-1 patient isolates. In contrast to chimpanzees, only one of the two GP120 variants has been used in humans for vaccination experiments up until now. Neither MN GP120 nor SF2 GP120 offers certain protection against infection by a virus variant such as occurs in a person infected by HIV-1 (patient isolate or wild-type virus). J. A. Levy offers in *"HIV and the Pathogenesis of AIDS"*, Publisher: Jay. A. Levy, Chapter 15, 2nd Edition, ASM Press Washington, D.C., 1998, an overview of the state of research in connection with vaccination with GP120 envelope protein.

The disadvantage of the vaccination strategies discussed or examined up until now in the prior art is, among other things, that, with the vaccines used, one is not in a position to prevent the formation of ever-new virus variants during the course of the viral illness.

The object of the present invention is therefore to provide a vaccine which in particular is in a position to prevent or clearly reduce the formation of new virus variants during the course of a viral illness and to limit or prevent the possibilities that the virus can escape the human immune defence and repeatedly infect new defence cells of the immune system.

In order to achieve the object, the items described in the following claims are proposed according to the invention.

The subject of the present invention is thus inter alia a protein vaccine which comprises a mixture of viral protein molecules, the molecules being sequence variants of a single viral protein or of part of same.

According to the invention, sequence variants of a protein are understood to be those molecules which contain an amino acid sequence derived vis-à-vis a native viral protein or a part (fragment) of same, the variants differing from each other in that at least one amino acid can be exchanged at any chosen point of the sequence or parts of same. The sequence variants preferably contain several amino acid exchanges at various points of the sequence, which are responsible for the production but also the bonding of virus-neutralizing antibodies. The number and position of the exchanged amino acids depends on the amino acid variability of the regions of the gp120 which are observed during the cell culture of adapted and wild-type HIV isolates. According to the invention, the sequence variants have a heterogeneity at at least two amino acid positions of the sequence or parts of same. A heterogeneity at three

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to eight and preferably at more than eight amino acid positions is particularly preferred, all amino acids occurring at these positions being possible. The number of possible sequence variants which represent the vaccine in their totality result from the combination of the various amino acids which are possible per position.

The invention relates in particular to a vaccine which comprises a mixture of $\geq 10^2$ sequence variants, i.e. a mixture of more than 10^2 molecules of various amino acid sequences (homologues). A vaccine is particularly preferred which comprises $\geq 10^3$ and preferably $\geq 10^4$ sequence variants.

Within the framework of the present invention it is assumed that the vaccine can comprise, along with the sequence variants, the protein per se from which the sequence variants are derived.

An anti-viral active ingredient is thus provided according to the invention which comprises virus-specific proteins which all differ in their sequences or parts of same. In order to achieve this, the protein-coding gene is newly synthesized in order to generate new cleavage sites for DNA-restriction enzymes, in order to permit the exchange of specific regions. By chemical synthesis of DNA fragments which code for the concerned protein section, a gene bank is then produced for the protein-coding sequence. The expression vectors which code for the protein, are then transfected as a mixture into cells. From these protein-producing cells the mixture of the various viral proteins

can then be isolated which according to the invention represents the preferred active ingredient.

In the case of HIV as a viral illness, the chronic infection and the associated continuous damaging of the immune system during the course of the illness lead to the complete loss of the specific virus defence. Neutralizing antibodies which have the property of forming a specific, very strong bond with certain antigenic structures are part of the specific virus defence. Foreign antigens are thereby marked and their interaction with e.g. virus receptors are blocked. Such a specific virus defence are neutralizing antibodies against the V3 loop of the external GP120 envelope protein. Such anti-V3 loop antibodies are in a position to prevent the infection of cells. In animal models, it has been shown that an infection by HIV-1 can be prevented by administering a specific monoclonal V3 loop antibody. By administering the same monoclonal antibody, it was also possible to heal an existing infection.

In contrast to experimental systems, the development of ever-new virus variants is however observed in the natural course of HIV infection. Thus hundreds of different V3 loop variants are found in a single patient at one time, as the variation of the HIV-1 is particularly high precisely in the V3 loop. The V3 loop is an important dominant antigenic domain of the GP120. A very specific humoral immune response is therefore formed against each V3 loop. The result of the induction of a highly specific immune response against the V3 loop is that neutralizing antibodies against the V3 loop of the HIV-1 variant A are not in a position to neutralize the variant B and vice

versa (Schreiber et al., J. Virol. 68 (1994) 3908-16, Abrahamsson et al., 4 (1990) 107-12).

In the asymptomatic phase of the HIV illness, the cell-free virus variants in the serum are neutralized by antibodies. Only later can virus variants be observed in the serum, which escape autologous neutralization. These V3 loop escape variants are no longer recognized by V3 loop antibodies and are thus no longer neutralized either. All other virus variants which are found in the serum of the patients are however recognized by autologous V3 loop antibodies. This indicates that during the course of the illness, virus variants occur against which no V3 loop antibodies exist. If the antibody content of serum samples of a patient is examined over a period of several years, the V3 loop antibodies against the V3 loop escape variant which occurs in the later stage can be detected in the serum samples which were taken at the beginning of the asymptomatic phase. The result is not, as with other infectious diseases, the standard escape of the pathogen through ever-new antigenic variation, but the switching-off of the neutralizing immune response to a virus replicating in the patient. The observed absence of the neutralizing V3 loop antibodies is thus the result of a continuous loss of the type-specific V3 loop antibodies. The corresponding virus can multiply through the loss of the neutralizing antibodies, which leads to an increase in the viral load in the serum of the patients. In the course of the illness, the increase in the viral load in the lymph nodes is also observed (Chun et al., Nature 387 (1997) 183-188).

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A virus which establishes itself against the immune system in the patient and develops into the dominant virus variant must necessarily overcome all antiviral barriers which the immune system offers. Along with neutralizing antibodies, cytotoxic T cells are also in a position to suppress the virus reproduction. Cytotoxic T cells effect the death of HIV infected CD4+ T cells. Along with the loss of the neutralizing antibodies, the loss of such cytotoxic T cells against HIV infected cells is also observed (Zerhoui et al., Thymus 24 (1997) 203-219; Gould et al., Semin Cell Dev Biol 9 (1998) 321-328; Wagner et al., J Gen Virol 74 (1993) 1261-1269; O'Toole et al., AIDS Res Hum Retroviruses 8 (1992) 1361-1368; Shearer et al., 137 (1986) 2514-2521). The object of a heterologous vaccine is therefore to induce or activate the mixture of many different GP120 envelope proteins of HIV both neutralizing antibodies and cytotoxic T cells against many different virus variants.

As already stated, the HIV illness is characterized in that the virus is constantly changing. This is effected by the error rate of the viral enzyme reverse transcriptase which commits errors during the increase of the viral genetic information. Mutants are thereby produced which are on the one hand selected because of their biological properties and the antiviral action of the human immune system. As with HIV, there are also other pathogens which transcribe their genetic information without error correction, which can lead to the formation of many variants. These include Hanta and Lassa viruses as well as hepatitis A, B and C viruses. Thus it is observed in people vaccinated for hepatitis B that vaccination fails in approx. 2%. In these

2%, hepatitis B "escape" variants are found which cannot be suppressed by the immune response induced by the vaccine.

The formation of vaccine, therapy and immune escape variants is based on the genetic variability of these viruses (Blum, Int J Clin Lab Res 27 (1997) 213-214; Jongerius et al., Transfusion 38 (1998) 56-59).

Up until now it has not been known in the state of the art how such a loss of virus-neutralizing antibodies can be countered. The strategies adopted to date were successful only in certain respects, i.e. in relation to the formation and the maintenance of individual variant-specific antibodies against specific viruses, but the treatment methods proposed to date in the form of antiviral medicaments or inoculants based on a memo-substance cannot counter the loss of type-specific antibodies against the variety of the protein variants formed by the virus in the course of the illness.

It is possible, through the present invention of a heterogeneous vaccine, to prevent the loss of neutralizing V3 antibodies or at least to clearly counter this loss.

On the basis of the present invention, for the first time an immunoreconstitutive treatment of virus-infected patients, in particular infected with HIV-1, is provided in which the immune system is activated such that the naturally acquired immune defence against the virus population of the various virus variants is regenerated and stimulated anew, in order to thus prevent the virus variants present in the patient from multiplying.

In short, the invention is based on the concept of preparing a mixture of GP120 proteins for an active ingredient against HIV-1, these GP120 proteins differing e.g. either in the V2 or the V3 loop or simultaneously in the two variable domains, i.e. the V2 loop and the V3 loop. In order to achieve this, the GP120-coding gene has to be newly synthesized accompanied by the formation of new (monovalent) cleavage sites for DNA-restriction enzymes which allow the specific exchange of the e.g. V2 and V3 region. By chemical synthesis of DNA fragments which code for the V2 loop and V3 loop a V2/V3 gene bank for GP120 is then produced. The GP120 expressions vectors are finally transfected into cells as a mixture, and the mixture of the various GP120 proteins which can be used as an active ingredient for the prophylaxis and/or therapy of the HIV illness or AIDS can then be isolated from these GP120-producing cells.

The consideration on which the present invention is based thus comprises the preparation for an immunotherapy or a vaccine against HIV-1 of many different GP120 molecules which carry V3 loop sequences (and/or V2 loop sequences) such as can also be identified in virus variants in patients.

The production of a mixture of natural virus variants in the cell culture system is very expensive. It must be borne in mind in particular that the composition of a virus mixture changes as cells which can be infected by HIV-1 have to be used in the cell culture. Some virus variants which have selective advantages, e.g. faster growth

kinesis, therefore become dominant in the cell culture system after only a few days and expel the other slower virus variants. This applies above all when the various virus variants which are present in the peripheral mononuclear blood cells (PMBC) or in the serum of patients are to be isolated. As it is not possible to cultivate a constant mixture of HIV variants, neither can a corresponding mixture of GP120 proteins be produced nor isolated in this way from virus cultures.

Within the framework of the present invention, a genetic-engineering approach was therefore chosen for the preparation of HIV-1 variants and GP120 variants. For the preparation of virus variants and recombinant GP120, a cloning system was constructed which consists of two vectors. The central component of both vectors is a novel, chemically synthesized HIV-1 envelope protein gene (HIV-1 env gene) which codes for the GP120 protein. One vector contains the entire genome of HIV-1. After transfection of this vector into cells, the cells produce infectious virus particles. This facilitates the production of a mixture of virus variants as cells can be used which are resistant to an HIV-1 infection. Therefore, in such a cell culture system, the composition of the virus mixture cannot be changed, because of the biological properties of the virus variants. The second vector facilitates the expression of GP120-variants in eukaryotic cells. This vector serves directly for the preparation of the inoculant (the vaccine).

For the genetic-engineering preparation and expression of the V3 loop GP120 variants in eukaryotic cells, a special

gene construct for HIV-1-env was created, called gene cassette in the following. For the preparation of the gene cassette, according to the invention the entire coding sequence of the env gene was chemically synthesized. With the help of this method it is possible to change the coding sequence of the gene as desired, new DNA-recognition sequences for DNA-cutting restriction enzymes being able to be incorporated into the env-gene sequence. It must be borne in mind that the amino acid sequence of the original GP120 (preferably of the strains NL4-3 and PI-932) does not change, while new restriction cleavage sites form in the DNA sequence of the env gene. At the same time, according to the invention cleavage sites for enzymes which repeatedly occur in the env sequence are removed so that in each case there is only one cleavage site for a specific restriction enzyme, such as e.g. BglII. According to a preferred version of the invention ten new, once-occurring (monovalent) cleavage sites are inserted at intervals of approx. 150 base pairs. The new env gene produced in this way is also called gene cassette within the framework of the present invention. In principle, the new env fragment is similar to a polylinker.

The cleavage sites for the restriction enzymes BstEII and BamHI define the gene cassette preferred according to the invention (SEQ ID NO: 9). In order to be able to exchange the region of the env gene, both cleavage sites are present only once in the pBSCenvATG expression vector (SEQ ID NO: 10) for gp120 and in retroviral vector pNL4-3. The vector, given the reference code pBSCenv-V3 by the depositor, was deposited on 6th January 1999 at the DSMZ - Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH,

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Mascheroder Weg 1b, 38124 Brunswick, Germany, under the accession number DSM 12612 in accordance with the Budapest Treaty.

Through the exchange of the gene section defined by BstEII and BamHI, the newly created sections of the env gene which are likewise defined by BstEII and BamHI, can be cloned into both vectors. The PstI/BclI V2 loop and the BglII/XbaI V3 loop env fragment are chemically or enzymatically synthesized and then cloned into a standard vector such as e.g. pUC 18 or 19. In this vector, the fragment is checked by sequencing and then cut out of the vector by a Pst/BcII or BgIII/XbaI digestion and transferred into the pBSCenvATG and then into the pNL4-3 vector. In place of the intermediate cloning into a standard vector (pUC 18, pUC 19 etc.), the PstI/BclI V2 loop and BglII/XbaI V3 loop fragments can also be cloned directly into pBSCenvATG.

As there is a cleavage site for a restriction enzyme every ca. 100 base pairs in the gene cassette, all regions of the env gene, in particular the V3 loop or V2 loop, can be exchanged for any DNA fragments. The section coding for the V3 loop can be exchanged for a BglII-XbaI fragment, which has a size of 244 base pairs (cf. SEQ ID NO: 9; nucleotides 708 to 955). The aim is to exchange the gene sections which code for the variable loops of the GP120 protein for new DNA fragments. The fragments are synthetically produced and then cloned into the env gene cassette. If the sequence is varied at certain positions during the synthesis of the V2 loop and V3 loop DNA fragments, i.e. all four nucleotides are incorporated equally or the nucleotide inosine is used at this position, env-gene variants with a pre-set

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variation of the thereby-coded amino acid sequence can be produced. The V3 loop sequences of patient isolates are the starting point for the preparation of the GP120 mixture. If these variants are cloned into the *gp120* gene cassette, the corresponding GP120 proteins can be expressed with the variable antigenic domains in eukaryotic cells. Sequence data of HIV-1 patient isolates are available for the variation of the V3 loop region of the *env* gene. Patient isolates are virus variants which have been cultivated direct from patient material, serum or infected cells from the blood in the laboratory. These viruses are characterized, unlike HIV-1 viruses which are cultivated in the laboratory for a long time, by particular properties. These viruses are more resistant to neutralizing antibodies and chemokines. In contrast to cell culture-adapted viruses, patient isolates use different co-receptors for the infection of cells. As patient isolates differ greatly from laboratory-adapted viruses, the collected V2 loop or V3 loop sequence data of the *env* genes of such viruses are to be used for the preparation of the GP120 mixture. Within the framework of the invention, additional base exchanges are possible in regions outside the sequence sections coding for V2 and V3.

According to a particular version of the invention, a protein vaccine is made available which comprises a mixture of GP120 proteins of the human immunodeficiency virus (HIV) which in each case differ from each another in their amino acid sequence in the region of the V3 loop and/or of the V2 loop.

Within the framework of the present invention, during the genetic-engineering preparation of the protein mixture or the protein vaccine, sequence variations are introduced preferably in regions of the V3 loops which lie outside consensus sequences (cf. e.g. M. Schreiber et al., J. Virol. 71 no. 12 (1997) 9198-9205). The consensus sequences are sequence sections which essentially remain both between different virus strains and during the formation of virus variants as a viral illness progress (HIV illness) in the body. Such a section is the sequence Gly-Pro-Gly-Arg-Ala-Phe (GPGRAPH) in the case of the HIV-1 of B sub-type. To the left and right of this sequence unit lie short regions 4-10 amino acids long which can vary greatly. In Fig. 1 this is represented using as example the V3 loop sequence variations of patient isolates.

In addition to variations in the region of the V3 loop, the molecules of the protein vaccine according to the invention can also contain sequence variations in the region of the V2 loop. Changes in the amino acid sequence outside consensus sequence regions are preferred in this case also (see Fig. 2). Furthermore, additional amino acid exchanges are possible in regions outside the V2 and V3 loops.

In connection with the present invention the normal one- or three-character codes are always used to describe the amino acids. When there is variation of the amino acid sequences inside the protein vaccine according to the invention, any desired amino acid exchanges are possible. In each case, however, amino acids of the viral GP120 sequence or of the sequences of the V2 loop and V3 loop are preferably exchanged for amino acids which also occur in other virus

variants at the corresponding sequence positions (see Fig. 2, position of 1997 sequence data in: Human Retroviruses and AIDS, A compilation and analysis of nucleic acid and amino acid sequences, Los Alamos National Laboratory, Los Alamos, NM 87545, U.S.A., Editors: B. Korber, B. Hahn, B. Foley, J.W. Mellors, T. Leitner, G. Myers, F. McCutchan, C. Kuiken).

Within the framework of the present invention, for the first time a protein vaccine is made available which is prepared by genetic engineering and comprises sequence variants of specific viral antigens (proteins). The preparation of the vaccine is described in the following using the V3 loop as an example. It is self-evident to the person skilled in the art that the synthesis principle described below can be transferred to other viruses or viral proteins or parts of their sequences.

In short, a mixture of GP120 proteins with variable V3 sequences which are modelled on the variability of the V3 loop of the GP120 protein of HIV is prepared as a basis for the vaccine.

For this, the sequence coding for the GP120 protein is firstly cloned piecewise into the plasmid pUC 18/19. This takes place through a polylinker exchange so that all interesting sections with monovalent restriction sites are flanked by the possible restriction sites which were previously inserted in the gp120 sequence by silent mutations, and thus can be subsequently cut out and exchanged.

Two homologous nucleic acid oligomers are then prepared with the help of a DNA synthesizer (length approx. 300 base pairs). After hybridization has taken place, the double-stranded DNA fragment forms with the DNA sequence of the V3 loop. Various standard methods from molecular biology can be used for the preparation of the double-stranded V3 loop DNA fragment. In one method, the variables are introduced by inosines and in the case of the corresponding complementary strand the variables are added by a nucleotide mix (AGCT, AGC, AG, ...). In this method, the DNA fragment is prepared by exclusively chemical means. In another method, a combination of chemical and enzymatic DNA synthesis, the variables are introduced by nucleotide mixtures. The oligonucleotides are then hybridized at complementary ends of a length of 20-30 bases, which are not variable. The synthesis to produce the completely double-stranded molecule takes place enzymatically using a DNA polymerase. Both isothermal DNA polymerases (Klenow fragment) or thermostable DNA polymerases (Taq-polymerase) can be used. If Taq polymerase is used, then larger amounts for the cloning of the V3 loop DNA fragment can also be prepared using the polymerase chain reaction. Using these methods, a double-stranded DNA fragment is prepared which is variable at specific positions. This degenerated DNA sequence codes for the corresponding variety of V3 loop amino acid sequences of the GP120 protein mixture to be prepared, the protein vaccine.

The mixture of the synthesized V2 fragments has a PstI cleavage site at the 5' end and a BclI cleavage site at the 3' end. The mixture of the synthesized V3 fragments has a BglII cleavage site at the 5' end and an XbaI cleavage site

at the 3' end. Using the two respective cleavage sites, the fragment mixture is cloned into a vector, e.g. pUC18 delta-env or pUC 18 BstEII-BamHI (Fig. 3). After this cloning a mixture or a pool of plasmid DNAs of the vector pUC 18 forms, which all have the complete BstEII-BamHI env fragment. All plasmid DNAs differ exclusively in the sequence of the env V2 loop or V3 loop. By inserting the V2 or the V3 fragments, the deletion (delta env) of the env gene section in the vector pUC18 is stopped.

This thus-prepared mixture of the plasmid DNA with variable env fragments is transformed into *E. coli* and fermented. *E. coli* amplifies and replicates this mixture of plasmid DNA. All possible variables of these V2/V3 loop plasmids are produced.

This plasmid pool is then isolated. The env fragment is then cut out of the mixture of the plasmid DNA by BstEII and BamHI digestion and cloned direct into the vector for the expression of the viral gp120. This vector has the advantage that the GP120 is also expressed in the eukaryotic cell system.

This pool of BSCenvATG-vector DNA with variable gp120 construct is now transfected into Cos or Chinese hamster ovary cells (CHO cells). These eukaryotes then express this pool of plasmids so that statistically the corresponding protein is translated to each variable and then - depending on which eukaryotes are used - correspondingly glycosylated. The proteins are then harvested, followed by purification to give the finished product (protein mixture or GP120 mixture with variable amino acid sequence).

By way of illustration, the process for producing the protein vaccine according to the invention is represented clearly in Fig. 4.

As already shown, variations in the V2 loop and/or in the V3 loop can be carried out. The variation of the V2 loop can take place according to the same scheme as described for V3.

The preparation of the protein vaccine according to the invention takes place, as described above, via various key constructs, i.e. nucleic acid intermediate steps and DNA constructs, which are essential for carrying out the invention.

As already explained, the nucleic acid sequence coding for the GP120 protein is cloned piecewise into pUC 18/19, the starting point according to the invention being a gp120 sequence in which the repeatedly present restriction sites are firstly modified by silent mutations such that each of the remaining restriction sites still occurs only once in the sequence. This sequence modification is necessary in order to be able to insert the expression cassette, which is necessary for producing the sequence variants, selectively at a quite specific point which is defined by two restriction sites each occurring only once in the sequence. Methods for the introduction of silent mutations in a nucleic acid sequence are well known to a person skilled in the art.

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A subject of the present invention is therefore also a nucleic acid sequence which is derived from the env sequence in SEQ ID NO: 1 or a fragment of same, it being modified such that it contains exclusively monovalent restriction sites. Preferably, the modification takes place by the introduction of silent mutations. According to a preferred version of the invention, the nucleic acid sequence has the sequence shown in SEQ ID NO: 9. The sequence of the gene cassette can be modified such that it contains the total env gene or parts of the env gene of virus isolates from patients. Preferably, such a gene cassette is to contain the env sequence of the patient isolate PI-932 (SEQ ID NO: 11). A gene cassette preferred according to the invention based on the PI-932 sequence is shown in SEQ ID NO: 12.

A subject of the present invention is therefore also a single-stranded nucleic acid sequence which contains the region coding for the V3 loop and/or for the V2 loop or fragments or parts of same in which, in the case of the V3 loop, a BglIII-XbaI (247 bp) or also a BglIII-NheI (283 bp) fragment can be exchanged for a modified fragment which carries nucleic acid exchanges or mutations at least 6, preferably at 9 to 20 positions, and in the case of the V2 loop, a PstI-BclI (139 bp) or also a PstI-EcoRI (339 bp) fragment for a modified fragment which carries nucleic acid exchanges or mutations at least 6, preferably at 9 to 20 positions. The nucleotides within a nucleic acid sequence are in each case replaced either by inosine or by a mixture of 2-4 nucleotides. This is explained using the example in Fig. 5. If various amino acids are to be combined at 7 amino acid positions of the V3 loop 21 to give 1152

variants, two nucleotides must be introduced by chemical synthesis at each of 11 nucleic acid positions into the sequence of the single-stranded nucleic acids (oligonucleotides) (Fig. 5).

The single-stranded nucleic acid sequences are - as already mentioned above - hybridized or synthesized to give the double strand.

The present invention therefore furthermore relates to double-stranded DNA which comprises hybrids of the above-mentioned single-stranded nucleic acid sequences, where in each case one nucleic acid sequence (5'-3' oligomer or 3'-5' oligomer) contains inosines at one or more selected positions, and the other nucleic acid sequence (3'-5' oligomer or 5'-3' oligomer) in each case contains two, three or four of the possible nucleotides (adenine, A; thymine, T; guanine, G; cytosine, C) at the corresponding complementary positions during the later hybridization. Sequences are thereby produced in which the four bases are present randomly distributed at the respective positions of the single-stranded nucleic acid sequence (DNA) so that the desired combinations of A, T, G or C are produced for the corresponding desired amino acid codons (a codon is formed from a series of three nucleotides). Because of the variation of nucleotide positions, randomly distributed sequence combinations result. Therefore, e.g. for the combination of (A, C) (ACT) (ACGT) a total of $2 \times 3 \times 4 = 24$ possible DNA sequences form, which code for various amino acids. At the same time, the number of variable positions also determines the number and position of the introduced inosines in the complementary DNA sequence. The calculation

of the heterogeneity of such a construct is shown in Fig. 5.

As, to form the double-stranded DNA, singled-stranded DNA containing inosines is in each case hybridized with oligomers containing A, T, G or C in each case randomly distributed at the corresponding positions, a mixture of double-stranded *gp120*-sequence or of part (fragment) thereof is obtained, the nucleic acid sequences in each case being derived from the *env* sequence (SEQ ID NO: 1 or 12) and the nucleic acid sequences in each case being distinguished from each other in the region coding for the V2 loop and/or in the region coding for the V3 loop. This means that nucleic acid sequences contained in the mixture differ from each other such that they code for a mixture of proteins which in each case contain amino acid sequences which are different from each other in the V2 loop and/or in the V3 loop.

According to a preferred version of the invention, in this way, at least 10^2 , preferably at least 10^3 and according to a particularly preferred version of the invention at least 10^4 sequence variants can be obtained at nucleic acid level (DNA level). This DNA mixture is hereafter called the DNA pool, and in relation to the variants of the *gp120* sequence this is hereafter called the pool with variable *gp120* construct (*gp120* pool).

Within the framework of the present invention, the term sequence variants of a DNA sequence is taken to mean molecules which have a derived nucleic acid sequence vis-à-vis a native viral DNA molecule or a part (fragment) of

same, the variants being distinguished from each other in that at least one nucleotide can be exchanged at any chosen points of the sequence. Preferably, the sequence variants contain several nucleotide exchanges at various points of the sequence, the number and the position of the exchanged nucleic acids essentially depending on the length of the nucleic acid sequence.

Upon the expression of the *env*-gene variants starting from the produced plasmid-DNA mixture, it is to be expected that, because of the random distribution, all possible DNA sequences are expressed. Therefore, all sequence variants that are possible, on the basis of the pre-set DNA mixture, of the *gp120* molecule or of part of same are then also formed. The heterogeneity of the *gp120* mixture is calculated on the one hand using the heterogeneity of the DNA sequence and the degeneration of the genetic code for the amino acids (cf. also Fig. 5).

The heterogeneity of the plasmid-DNA mixture is demonstrated by DNA sequencing of single clones. To this end, the mixture of the plasmid DNA is transformed in *E. coli* and single clones are randomly selected and their V2 or V3 loop sequence determined. In total, approx. 100-200 different clones are to be sequenced. The statistical distribution of the DNA sequences can be used to calculate the distribution of the whole mixture. The method corresponds very largely to the method of random sampling such as is used as standard for the quality control of the most varied products.

The direct molecular analysis of the heterogeneity of the GP120 protein mixture proves to be somewhat more difficult, as single GP120 molecules cannot be separated from the mixture and identified. This is in the nature of things, as the individual GP120 variants differ from each other in a few amino acids only. Gel electrophoresis separation or mass spectroscopic analysis can establish whether a mixture or a single form of the GP120 molecule is involved. When the GP120 mixture is used e.g. in animals as a vaccine, the immune response induced in the animal is directly dependent on the number and the composition of the GP120 mixture. The immune response of the animal, the neutralizing antibodies can then be examined in virus neutralization tests. To monitor a corresponding variant-overlapping immune response, various patient isolates of HIV-1 can be used in the neutralization test. Preferably, patient isolates are chosen which differ in the ability to use various co-receptors for the infection of the target cells. The neutralization potential of the GP120 mixture then serves as a quality measure.

A subject of the present invention is therefore furthermore a protein mixture which comprises GP120 proteins which contain amino acid sequences which in each case differ from each other in the V2 loop and/or in the V3 loop, the mixture containing, according to one version of the invention, at least 10^2 , preferably at least 10^3 , according to a version of the invention, and, according to a particularly preferred version of the invention, at least 10^4 sequence variants.

As already mentioned earlier, the mixture of double-stranded DNA which can be obtained by hybridization of inosines-containing single-stranded DNA with randomly distributed A-, T-, G- and/or C-containing single-stranded DNA, i.e. the pool with variable GP120 construct, is transformed and fermented in prokaryotic or eukaryotic host cells, preferably *E. Coli*.

A subject of the present invention is therefore plasmids which contain inserted double-stranded DNA, in each case comprising hybrids of the single-stranded, inosine-containing nucleic acid sequence (see above) with the single-stranded nucleic acid sequence containing a mixture of all four nucleotide variants (A, T, G, C) (see above). Furthermore, the invention relates to a vector mixture which contains a mixture of these plasmids, the nucleic acid sequences of the plasmids in each case differing from each other in the region coding for the V3 loop and/or in the region coding for the V2 loop. According to one version of the invention, the vector mixture comprises at least 10^2 , preferably at least 10^3 and according to a particularly preferred version of the invention at least 10^4 of the named plasmids. Depending on which host cells are to be used to express the vectors (plasmids), various expression systems and base vectors well-known to a person skilled in the art can be considered (cf. Methods in Enzymology, Vol. 185, Gene Expression Technology, 1991, Publ. D.V. Goeddel, Academic Press, Inc.). Thus, for the expression in *E. coli*, the plasmid pUC 18/19 is preferred as base plasmid into which the nucleic acid sequence variants are cloned. Within the framework of the present invention, the vector mixtures can thus be expressed either in bacterial host cells such

as *E. coli*, or in eukaryotic host cells, preferably from the group consisting of Cos, CHO or baby hamster kidney cells (BHK cells) or in other host cells.

The present invention thus furthermore relates to *E. coli* host cells or eukaryotic host cells which are transfected with a vector mixture according to the invention.

As host cells are transfected with the DNA pool (the vector mixture), it is to be expected that as great or at least approximately as great a number of differently transformed host cells forms as there are sequence variants present in the DNA pool. Upon the preparation of the vaccine, special attention is to be paid to the yields during the individual cloning steps. The yields upon preparation of the double-stranded DNA fragments, the ligation of the V3 loop and V2 loop DNA fragments into the *env* gene and the transformation or the preparation of bacteria and cell clones must be such that the heterogeneity that is to be achieved of the mixture is not restricted. This is to be illustrated using an example. If, e.g. a number of balls are to be removed from a quantity of balls which differ on the basis of two colours, but there is to be a 99.9% probability that both colours are present in this selection, approx. 13 balls would have to be removed (G. Schreiber, Ein kombinatorisches Problem aus der Genetik; Bio-engineering 1988, 2: 32-35). Transferred to the cloning steps of the HIV vaccine, this means: If a heterogeneity of approx. 6000 virus variants is to be produced, 13 times as many clones would have to be produced in each individual cloning step. Thus, a gene bank of approx. 80,000 clones must be prepared for the V3 loop. Starting from this gene bank, the

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The present invention also relates to a process for the preparation of a nucleic acid sequence coding for a viral protein (expression cassette) in which the sequence is so modified, or preferably so many silent mutations are introduced, that it then contains at least two and preferably exclusively monovalent restriction sites. Particularly preferably, the sequence still contains only monovalent restriction sites. Preferably, the protein coded by the nucleic acid sequence is GP120, the env wild-type sequence coding for the GP120 being varied by silent mutations.

Within the framework of the present invention, a process is furthermore made available for the preparation/production

Finally, according to the invention, a process is for the first time made available for the preparation of a DNA vaccine, in which the process according to the invention for the preparation of the vector mixture is carried out, the plasmids according to the invention expressing the mixture of the *gp120* proteins after application in host cells (e.g. human and animal monocytes). The DNA vaccine can optionally be formulated with pharmaceutically compatible auxiliaries and/or carriers for application and after administration in the organism, make possible the production of the sequence variants of viral proteins.

The present invention thus also relates to a pharmaceutical composition or a DNA vaccine which codes for a mixture of structurally different virus proteins, the vaccine containing a mixture of sequence variants of a viral DNA molecule or of part of same, i.e. of DNA molecules the nucleic acid sequences of which differ from each other in the region coding for the protein, a part or a fragment of same. By the term "structurally different virus proteins" is meant according to the invention those proteins the amino acid sequences of which are derived from the wild-type sequence of the corresponding virus protein, the amino acid sequences deviating from each other insofar as they

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differ from each other and compared with the wild-type sequence through one or more exchanged amino acids at identical or different positions of the sequence. As already mentioned, the nucleic acid sequences in the vaccine code preferable according to the invention for a mixture of structurally different GP120 proteins of HIV (sequence variants of GP120), a vaccine being particularly preferred which contains a mixture of DNA molecules, the nucleic acid sequences of which differ from each other in the region coding for the V2 loop and/or in the region coding for the V3 loop of *gp120* of the HIV-1. In this context, reference is also made to Fig. 3 in which known structural differences or variations in the V3 loop of GP120 are shown.

The DNA vaccine according to the invention is of particular importance within the framework of a gene therapy.

Finally, according to the invention, a process for the preparation of a pharmaceutical composition or a protein vaccine is for the first time made available in which the host cells according to the invention, i.e. host cells which are transformed with a vector mixture according to the invention, the vectors in each case containing plasmids, the nucleic acid sequences of which differ from each other in the region coding for the V2 loop and/or in the region coding for the V3 loop, are cultivated under conditions which allow the expression of the mixture of viral protein sequence variants. The host cells are preferably bacterial host cells, such as *E. coli*, or eukaryotic host cells, preferably from the group consisting

of Cos, Chinese hamster ovary (CHO) or baby hamster kidney cells (BHK cells).

Through the present invention, it is thus possible to make available a vaccine comprising variable GP120 proteins which, in the prophylactic and/or therapeutic treatment of a HIV infection or AIDS

1. activates the immune system,
2. induces the formation of HIV-1-neutralizing antibodies,
3. prevents the loss of neutralizing antibodies and
4. in which monitoring of the stimulation of the GP120-specific immune response is taken over by the active ingredient.

This is preferably carried out in that, even before the loss of HIV-neutralizing antibodies associated with the outbreak of AIDS, sufficient antigens are available which, because of their variety of different amino acid sequences (i.e. their sequence variations) are in a position to induce the formation of those HIV antibodies which normally, i.e. without corresponding prophylaxis according to the present invention, are lost or experience a reduction in concentration as the illness progress. In addition to a preventative treatment, the present invention also relates to therapeutic treatment, the GP120 mixture (protein vaccine) being used, to counter a reduction or a loss of HIV-neutralizing antibodies by activation of the immune system and induction of the formation of new or additional HIV-1-neutralizing antibodies.

According to the invention, the mixture of structurally different viral proteins which are sequence variants of a viral protein (preferably of GP120) or of part of same is therefore used for the preparation of a vaccine for the prevention and or therapy of a viral infection in humans. Furthermore, the present invention relates to the use of a mixture of DNA molecules which code for sequence variants of a viral protein or of part of same for the preparation of a vaccine for the prevention and/or therapy of a viral infection in humans. According to a preferred version, the invention relates to the use for the preparation of a vaccine for the prevention and/or therapy of a HIV infection in humans.

Within the framework of the present invention, the virus infection can be any infection in which self-replicating virus variants form in the course of the illness, where those amino acid sequence sections or nucleic acid sections coding for the latter can be considered according to the invention as selected protein sequences or protein-coding nucleic acid sequences, in which, in the course of viral illnesses, variants, i.e. sequence variations, are always or often observed. Preferably, in the present case, these are sequences variants of the GP120 molecule (at protein level) or of the region coding for GP120 (at DNA level) or parts of same, in particular sequence variants in the V3 loop or in the V2 loop, preferably both in the V3 and in the V2 loop.

According to the invention, pharmaceutical compositions or vaccines are consequently made available which, in the immunoreconstitutive treatment of those suffering viral

infection, activate the immune system in such a manner that the naturally acquired immune defence is regenerated against the virus and restimulated to thus prevent the virus variants replicating in the patient from multiplying. According to a preferred version of the invention, vaccines for the immunoreconstitutive treatment of those infected by HIV-1 are made available for the first time.

Within the framework of the present invention, the vaccines can either be formulated or administered per se, i.e. as DNA and/or protein mixtures without further additives, or together with further active ingredients, such as e.g. immunostimulants such as interleukin-2, CC and CXC-Chemokine, and/or pharmaceutically compatible auxiliaries and carriers. As a rule, the vaccines according to the invention are formulated for intravenous application, such as e.g. by the intravenous, intramuscular, subcutaneous routes. In addition, the vaccines can be formulated for oral, mucosal (intravaginal, intrarectal) and transdermal application.

The present invention has the following advantages:

Through a selective genetic manipulation of the nucleic acid sequence, with the help of a gene cassette, any desired variants of a pathogen or of a gene of the exciter can be prepared. The variations preferably affect the regions against which the neutralizing antibodies or cytotoxic T cells are formed. An inoculant in the form of a mixture of variants of the pathogen or of variants of the corresponding antigens of a pathogen can thereby be prepared. The advantages of this concept in relation to HIV

as a pathogen lie in the preparation of a mixture of HIV-GP120, the external membrane protein of HIV, against which the virus-neutralizing immune response of humans is directed. As, in the precise case of the HIV illness, each patient is exposed to a variety of HIV variants which all differ in the sequence of the GP120 protein, the use of a variety of GP120 variants as inoculant is of particular advantage, this GP120 mixture being able to be used both for the immunization of normally healthy persons and for the therapy of persons already infected by HIV. In the case of immunization with the GP120 mixture, as broad as possible an immune response against as many virus variants as possible in humans is induced, which in the ideal case protects against all HIV variants. In the case of therapy with the GP120 mixture, the loss of neutralizing antibodies and the loss of the HIV-specific cellular immune response can be combated.

According to the invention, it is furthermore possible for the first time to prepare *gp120* clones which, in the nature or variety of the observed sequence variations, correspond to plasma isolates of those infected with HIV or ill with AIDS (cf. M. Schreiber et al., J. Virol. 68 No. 6 (1994) 3908-3916). In this context, in contrast to various approaches followed to date, no isolated GP120 molecule or an antigenic fragment of same is used for immunization, but a protein mixture which consists of a variety of sequence variants which are characterized by a complete GP120 sequence which in addition contain the correct tertiary structure corresponding to the natural plication of the GP120 molecule. The conformation of the virus variants made available in the vaccine is important insofar as sequence

and structure variants are thereby provided which, with the GP120 variants detectable in the course of the viral illness, as great as possible a similarity with regard to the observed sequence variations and also the conformation required for the bonding to CD4 and the co-receptor is achieved, whereby an effective immune stimulation can be achieved.

Through the invention, a mixture of GP120 proteins is provided which represents the protein vaccine. At the same time, a mixture of the genes coding for this protein mixture is provided through the invention. These genes can be transferred into a vector which is suitable for direct application in humans (DNA vaccine) (Ulmer et al., 1995 Ann NY Acad Sci 772:117-125; Donnelly et al., 1995 Ann NY Acad Sci 1995 772:40-46). A DNA vaccine has the advantage that the heterogeneity of the mixture of the DNA vectors can be greater than that of the mixture of the recombinant GP120 proteins. It is easier to produce a high heterogeneity of a DNA vector mixture. Such a DNA vaccine would cover a much broader spectrum of HIV variants. From the technical point of view, the preparation of the DNA mixture is easier. A precondition for the DNA vaccine is a *gp120* expression vector which carries the two cleavage sites BstEII and BamHI. This makes possible the conversion of the V3 loop and V2 loop variables env gene fragments into such a vector.

With regard to the GP120 proteins, it is to be borne in mind that 5 potential points for N-glycosylation are located in the region of the V3 loop. The sugar groups protect the V3 loop against recognition by neutralizing

antibodies. Virus variants which contain a glycosylated V3 loop are more poorly neutralizable than virus variants in which glycosylation is incomplete. As virus variants with a complete V3 loop glycosylation can escape the neutralizing immune response, they are important for the transfer and for the establishment of the infection. In the course of the illness, if a part of the neutralizing antibody response is lost, virus variants whose V3 loop is not completely glycosylated establish themselves. As the V3 loop is no longer covered by sugar groups, these virus variants replicate faster than the completely glycosylated V3 loop mutants. According to the invention, it is therefore advantageous to take the varying glycosylation of the GP120 V3 loop into account when constructing the GP120 vaccine.

Within the framework of the present invention, the principle represented with reference to HIV can be transferred to other viruses which also form variants in the course of the viral illness. In particular, vaccines can be provided against a variety of viruses which induce a broad immune response against many virus variants in humans which in the ideal case protects against all variants. In the case of therapy with such vaccines, the loss of neutralizing antibodies and the loss of the HIV-specific cellular immune response can be effectively combated. In this context, it is furthermore possible or even advisable, with pathogen types (viruses) which form several sequence variants of several proteins in the course of an illness, to provide several gene cassettes according to the invention each of which contains the nucleic acid sequence coding for variants of each of these proteins or fragments

of same, in order to combat the loss of neutralizing antibodies against all conceivable virus variants.

Within the framework of the present invention, the advantages of a protein vaccine and a DNA vaccine can be advantageously combined in order to increase the success of a preventative and/or therapeutic treatment of a viral illness. A pharmaceutical composition is thus furthermore provided for the prevention and/or therapy of a viral infection, which comprises a protein mixture and a nucleic acid mixture, the protein mixture comprising sequence variants of a viral protein or of part of same, and the nucleic acid mixture comprises DNA molecules which code for sequence variants of a viral protein or of part of same. In particular, the pharmaceutical composition is a combination preparation which comprises both a protein mixture comprising one of the above-named sequence variants of the GP120 protein and a nucleic acid mixture derived from an above-named env sequence in SEQ ID NO: 1 or SEQ ID NO: 11 or a fragment of same.

The present invention is explained in the following, using examples, figures and a sequence protocol.

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- 2.21 Preparation of double-stranded DNA with the help of oligonucleotides
- 2.22 Transfection of COS cells and CHO cells
- 2.23 Chromatographic purification of the GP120 mixture
- 2.24 Preparation of the DNA vaccine

1. General description of the preparation process

1.1 Cloning of a mixture of V3 loop *gp120* variants

The oligonucleotides are synthesized as described under 2.6. As V3 loop sequence, a mutated sequence of the HIV-1 patient isolate F1-01 (M. Schreiber et al., J. Virol. 68 no. 6 (1994) 3908-3916) is given by way of example. Any other sequence or mixture of sequences is also possible. To clone many different variants of a V3 loop sequence, mixtures are used instead of pure nucleotide components at specific positions of the sequence. In this way, a mixture is obtained of oligonucleotides which all differ in the sequence and thus code for different V3 loops. Such oligonucleotide mixtures are also called oligonucleotides with degenerated sequences. Starting from chemically synthesized oligonucleotides with degenerated sequences, the region coding for the various V3 loops is shown.

An oligonucleotide, in reading-frame orientation (forward), is synthesized for the *env*-sequence section from the BglIII cleavage site to the first degenerated position. A second oligonucleotide, in complementary orientation (reverse), is synthesized according to the sequence from a position which lies 15 bases before the beginning of the variable region (method 2.6). Through overlapping of the 3' region along a

length of 15 bases in total, the hybridization of both oligonucleotides is carried out. In a subsequent reaction with DNA polymerase (e.g. Taq DNA polymerase or Klenow fragment), a completely double-stranded DNA molecule forms from the two hybridized oligonucleotides (method 2.21).

The thus-obtained DNA mixture is digested with restriction endonucleases BglII and XbaI. The cloning of the DNA mixture is carried out in the expression vector Δ V3 pBSCenvV3 cleaved with BglII and XbaI (methods 2.15 and 2.16).

This vector contains the coding sequence of the *gp160* of the HIV-1 strain NL4-3 (IIIB). The NL4-3 *env* gene was manipulated such that it possesses the restriction cleavage sites BglII and XbaI as well as ApaLI, PstI and BclI once each only. The region coding for the V3 loop, which lies between the cleavage sites BglII and XbaI, was removed and replaced by a 15 base pair sequence, whereby an analytical cleavage site was introduced for the enzyme AscI. The BglII- and SbaI- cleaved V3 loop DNA mixture is cloned into this vector (method 2.19). The loss of the AscI cleavage site in the finished V3 loop pBSCenvV3 vector can be used for the selection of the V3 loop coding clones (method 2.18).

The thus-formed plasmid mixture is transformed in DH5 α bacteria (method 2.12), a transformation rate of $>10^5$ having to be achieved. A gene library of V3 loop fragments with a size of approx. 10^5 clones is thereby obtained. The mixture is to be analyzed by DNA sequencing (see 1.3). With the help of this process, a mixture of clones is obtained,

which all code for different V3 loop variants of the GP120 protein. This mixture of vectors serves as starting product for the preparation of the protein mixture for use as inoculant and as starting product for use as DNA vaccine.

For the preparation of the GP120 protein mixture, the pBSCenvV3 expression vectors are transfected into COS cells and CHO cells (method 2.22). The purification of the different GP120 proteins, the active ingredient mixture, is carried out according to method 2.23, as described in the literature.

1.2 Cloning of a mixture of V2 loop *gp120* variants

Variants of the V1 loop and V2 loop are prepared in the same way. The expression vector $\Delta V3$ -pBSCenvV3 has three additional restriction cleavage sites for this. The variation of the V1 loop takes place through cloning into the *Apa*LI and *Pst*I cleavage sites. The variation of the V2 loop takes place through cloning into the *Pst*I and *Bcl*I cleavage sites.

1.3 Analysis of variability

The analysis of the V3 loop mixtures takes place through DNA sequencing (method 2.20). The statistical selection of approx. 100-200 clones whose V1, V2 and V3 loop sequences are to be determined, is planned. If all these clones are different, the heterogeneity of the gene library and thus also the heterogeneity of the protein mixture can be measured with the help of a statistic calculation.

2. Material and methods

2.1 Abbreviations

2.1.1 General abbreviations

µg microgram
µl microlitre
µmol micromole
APS ammonium persulphate
bp base pairs
dNTP desoxynucleoside triphosphate
DNA desoxyribonucleic acid
DTT dithiothreitol
EDTA ethylenediaminetetraacetic acid
g gram
GP glycoprotein
h hour
HIV human immunodeficiency virus
IPTG isopropyl-β-D-thiogalactopyranoside
MOPS 3-morpholinepropanesulfonic acid
OD optical density
PAGE polyacrylamide gel electrophoresis
PCR polymerase chain reaction
RT room temperature
TEMED N,N,N',N'-tetramethylethylenediamine

2.1.2 Nucleic acids

A adenine
C cytosine
G guanine
T thymidine

2.2 Bacterial strains

Escherichia coli DH5 α : F-, endA1, hsdR17,
(rk-mk+), su-pE44, recA1,
 λ -, gyrA96, relA1, Φ 80d lac
z Δ M15

2.3 Plasmids

pBSCenvATG own design, the sequence is given in
SEQ ID NO: 10.

2.4 Enzymes

Restriction enzymes MBI-Fermentas, Gibco-BRL, Biolabs
DNA polymerases MBI-Fermentas, Gibco-BRL
T4-DNA ligase MBI-Fermentas

2.5 Chemicals

[α -35S]dATP	Amersham Life Science
Agarose, ultra pure	GIBCO BRL
Ammonium persulphate	Merck
Ampicillin	US Biochemical
Bacto-Agar	Becton Dickinson
Bacto-Trypton	Becton Dickinson
Boric acid	Merck
Bromphenol blue	Merck
Calcium chloride	Merck

Desoxyribonucleotides	MBI-Fermentas
Dithiothreitol	Biotechnik, St. Leon-Rot
Glacial acetic acid	Merck
Ethidium bromide	Sigma
Ethylenediaminetetraacetic acid	Merck
Glycerine	Merck
Urea	ICN Biomedicals
Yeast extract	Becton Dickinson
Potassium chloride	Merck
Potassium dihydrogen phosphate	Merck
Magnesium chloride	Merck
Acrylamide mix	Roth
Sodium acetate	Merck
Sodium chloride	Merck
Disodium hydrogen phosphate	Merck
Sodium dihydrogen phosphate	Merck
2-propanol	Merck
Sigmacote (chlorinated polysiloxane)	Sigma
N,N,N',N'-tetramethylethylenediamine	Merck
Tris(hydroxymethyl)-aminomethane	GIBCO BRL

2.6 Oligonucleotides

All oligonucleotides listed were prepared with the ExpediteTM nucleic acid synthesis system of PE Biosystems (Weiterstadt). Oligonucleotides are used for the cloning of

env genes which differ from each other only in the sequence for the V3 loop. The sequences of the oligonucleotides are listed taking as an example the cloning of the V3 loop region for the HIV-1 patient isolate F1-01.

V3 loop: for cloning into pNL4-3/BglIII-NheI, F1-01,
forward:

5'-AAG ATG TAG TAA TTA GAT CTG CCA ATT TCA CAG ACA ATG CTA
AAA CCA TAA TAG TAC AGC TGA ACA CAT CGT TAG AAA TTA ATT GTA
CAA GAC CCA ACA ACA AT ACA-3' (SEQ ID NO: 3)

V3 loop: for cloning in pNL4-3/BglIII-NheI, F1-01:

5'-TTT TGC TCT AGA AAT GTT ACA ATG TGC TTG TCT TAT GTC TCC
TGT TGC AGC TTC TGT TGC ATG AAA TGC TCT CCC TGG TCC GAT ATG
GAT ACT ATG-(GA) (AT) (GATC) TTT TCT TGT ATT GTT GTT GGG-3'
(SEQ ID NO: 4)

For sequencing of V3 loop clones, the oligonucleotides 7010, 7011 and the M13 standard primer were used (M13, M13r).

Primer 7010: 5'-CCA TGT ACA AAT GTC AG-3' (SEQ ID NO: 5)

Primer 7011: 5'-AAA ACT GTG CGT TAC AA-3' (SEQ ID NO: 6)

M13-Primer: 5'-GTA AAA CGA CGG CCA GT-3' (SEQ ID NO: 7)

M13r-Primer: 5'-CAG GAA ACA GCT ATG AC-3' (SEQ ID NO: 8)

2.7 Molecular weight standards

1 kb conductor MBI-Fermentas

100 bp conductor MBI Fermentas

100180-65161660

2.8 Reagent kits used

T7 Sequencing-Kit	Pharmacia
Qiaquick PCR purification Kit	Qiagen
Qiagen Plasmid Kit	Qiagen

2.9 Media

YT Medium:

- 10g trypton
- 5g yeast extract
- 5g NaCl

dYT medium:

- 16g trypton
- 10g yeast extract
- 5g NaCl

dYT agar plates:

- 10g trypton
- 5g yeast extract
- 5g NaCl
- 15g agar

The quoted quantities relate in each case to 1000 ml deionized water. The mixtures were autoclaved at 121°C and 1.5 bar for 20 mins. Should the media contain antibiotics or other heat-sensitive reagents, the corresponding quantities were sterile-filtered and added after cooling of the medium.

Ampicillin 3.3 ml/l (60 mg/ml)

IPTG	3 ml/l (100 mM)
xgal	3 ml/l (2% in DMF, do not filter)

2.10 Sterilization of solutions and apparatus

Solutions and media as well as pipette tips, Eppendorf vessels and glass apparatus were autoclaved for 20-40 mins at 121°C and 1.5 bar. Heat-sensitive solutions such as e.g. antibiotics and IPTG solutions were sterile-filtered.

2.11 Cultivation and storage of bacteria

Bacteria cultures were in each case inoculated with a single colony. To isolate a single colony, cells of a liquid culture were spread on an agar plate. After incubation at 37°C overnight, isolated colonies have grown. For the short-term preservation of the bacteria, the agar plates were sealed with parafilm and stored at 4°C. For a long-term storage of bacteria strains, 0.75 ml of a YT-overnight culture were mixed with 0.25 ml sterile glycerine, shock-frozen in liquid nitrogen and stored at -70°C.

2.12 Preparation of competent cells

100 ml YT-medium were inoculated with 100 µl of an overnight culture. The bacteria were incubated until an $OD_{550}=0.4$ at 37°C was reached in the shaking incubator and then centrifuged for 8 mins at 4°C and 1000g. All subsequent work was carried out on ice using pre-cooled vessels and 4°C cold solutions. The cells were resuspended in 50 ml 50 mM $CaCl_2$ and incubated for 30 mins on ice. After

renewed centrifuging, the bacteria were taken up in 2.5 ml sterile TFBII buffer and divided into portions of 100 μ l each. The 100- μ l portions of the competent cells were able to be, insofar as they were not required for an immediate transformation, stored in liquid nitrogen at -70°C after shock-freezing.

TFBII buffer:

10 mM	MOPS pH 7.0
75 mM	CaCl_2
10 mM	KCl
15%	glycerine

2.13 Transformation of *E. coli*

(Hanahan, J. Mol. Biol. 166 (1983) 557-580)

A 100 μ l portion of competent cells was thawed in an ice bath and incubated with 1-100 ng plasmid-DNA for 30 mins at 0°C . The transformation mixture was then incubated for 1 min at 42°C . 1 ml YT-medium was then added and agitated for an hour at 37°C . To make possible a selection of the transformed cells, 100-500 μ l of the mixture were spread onto antibiotic-containing YT agar plates. After incubation overnight at 37°C , only colonies which carry the plasmid-coded resistance gene have grown. Upon a transformation of cells which do not contain any functional β -galactosidase (lacZAM15 mutation, e.g. DH5 α), vectors such as the pUC used make possible a direct selection (*blue white screening*) of recombinant bacteria via a plasmid-coded β -galactosidase. For the blue-white selection, the bacteria were spread onto Amp/IPTG/Xgal-YT agar plates. The substrate Xgal is cleaved into a blue dye by the β -

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galactosidase. Because of the destruction of the reading frame of the lacZ gene by the insertion of a foreign DNA fragment into the lacZ gene, white colonies are produced. On the other hand, blue colonies mean that the lacZ gene has remained functional during cloning and ligation and no DNA was inserted.

2.14 Plasmid DNA preparation

(Qiagen, Hilden)

To isolate plasmid-DNA from *E. coli*, the QIAprep Spin Miniprep Kit from Qiagen was used. The DNA preparation took place according to the manufacturer's instructions (QIAprep Plasmid Handbook 03/95). Plasmid-containing *E. coli* bacteria were inoculated in dYT-amp medium and agitated overnight at 37°C. Three ml of the *E. coli* overnight culture were used for the plasmid isolation. The bacteria were harvested (1 min, 14,000 rpm, Heraeus centrifuge) and taken up in 250 µl P1 buffer. The bacteria were decomposed through alkaline lysis (250 µl, 0.2N NaOH/1%-SDS). The mixture was neutralized through the addition of 3M potassium acetate, (350 µl, pH 5.5). The purification of the plasmid DNA is based on the selective bonding of plasmid DNA to DEAE anion exchanger columns. With the chosen salt concentrations and pH conditions, the plasmid DNA bonds to the DEAE matrix. The DEAE matrix was washed (750 µl, PE buffer, Qiagen). The plasmid DNA was then eluted with 50 µl H₂O and stored at -20°C.

To obtain plasmid DNA in a larger quantity (approx. 100 µg), 25 ml overnight culture were used. To purify the DNA, the QIAGEN Plasmid Midi Kit was used. The purification is

based in this case on the principle described under the "QIAprep Spin Miniprep Kit" and was carried out according to the manufacturer's instructions (QIAGEN Plasmid Purification Handbook 01/97).

2.15 Separation of DNA in agarose gels

The separation of DNA took place through gel electrophoresis in agarose gels. Depending on the size of the fragments examined, 0.8-2% agarose were dissolved by boiling-up in TBE buffer (for analytical agarose gels) or TAE buffer (for preparative agarose gels). After cooling to approx. 60°C, the gel liquid was mixed with 1 µl ethidium bromide (10 mg/ml) and poured into a prepared gel bed with inserted comb. The completely cooled gel was coated in an electrophoresis chamber with TBE or TAE buffer and the comb removed. The DNA samples were mixed with 1/5 vol. coating buffer and pipetted into the sample bags. The fragments were separated at 5-10 volt/cm gel length for 0.5-2 h. For detection, the gel was photographed after the electrophoresis in UV transmitted light. The molecular weights of the DNA bands were measured compared with following DNA molecular weight markers.

TBE buffer:

100 mM	Tris
100 mM	boric acid
3 mM	EDTA

TAE buffer:

40 mM	Tris
2mM	EDTA

0.114% glacial acetic acid

Coating buffer:

0.25% bromphenol blue
0.25% xylene cyanol
30% glycerine
50 mM EDTA

2.16 Purification of DNA from agarose gels

For the extraction of the DNA from agarose gels, the "QIAquick Gel Extraction Kit" (Qiagen, Hilden) was used. The buffer conditions were selected such that the nucleic acids bond to the silica membrane of the columns, whilst low-molecular-weight bacteria components pass through the membrane (method 2.15). The process was carried out according to the "Qiaquick Gel Extraction Kit Protocol" of the QIAquick Spin Handbook 07/97. The purified DNA was in each case eluted from the silica membrane with 50 μ l H_2O .

2.17 Separation of DNA in polyacrylamide gels

The separation of radioactively marked DNA fragments for the DNA sequencing was carried out using denaturizing polyacrylamide gels. Two purified glass plates were cleaned of fatty residues with ethanol and coated with 1 ml Sigmacote per plate to obtain a hydrophobic surface. Through the coating, a later tearing of the gel upon removal from the glass plate was to be avoided. Spacers were placed between the two glass plates, and served at the same time for lateral sealing. The whole apparatus was fixed with several clamps. To prepare the sequence gel, 21

g urea were dissolved in approx. 20 ml water. After the addition of 7.5 ml acrylamide mix and 5 ml 10x TBE buffer (see 2.15), the mixture was made up to 50 ml with water. The polymerization of the gel was started by the addition of 300 μ l APS and 100 μ l TEMED. Immediately afterwards, the gel solution was poured into the prepared gel apparatus and the pocket bottom shaped by inserting the flat back of the saw-tooth comb. The gel was stored horizontally at room temperature until polymerization was complete. After the insertion of the gel into the gel chamber, the latter was filled with TBE buffer. By rotating the saw-tooth comb, the deposit pockets were formed. A 20-min pre-run was carried out in order to heat the gel to the optimal operating temperature. The pockets were washed thoroughly with TBE buffer and then filled with 4-5 μ l of the samples. The electrophoresis took place at 2 kV (150 watt) for approx. 2-3 h. The gel was removed from the gel chamber after the electrophoresis was ended and one of the glass plates raised carefully. By laying-on and light pressing, the gel was transferred onto Schleicher & Schuell paper (Whatman, England). After drying at 80°C under vacuum, the gel was exposed on an X-ray film (Kodak BioMax MR-1, Sigma Deisenhofen) for 1-3 days at RT.

Acrylamide mix:

- 40% polyacrylamide
- 0.8% bisacrylamide

2.18 Cleaving of DNA

Restriction endonucleases recognize and hydrolyze enzyme-specific palindromic DNA sequences of mostly 4-8

nucleotides' length. Upon hydrolysis, depending on the type of enzyme, blunt ends or overhanging ends of single-stranded DNA form. For a restriction digestion, 0.1-60 µg DNA were incubated for 2-5 h at 37°C with 2-120 units of a restriction enzyme in the buffer indicated by the manufacturer. The total volume was between 10 µl for analytical and 300 µl for preparative mixtures. For restrictions with two different enzymes, a buffer was chosen in which both enzymes have a sufficient activity, e.g. the EcoRI buffer for an EcoRI/BamHI digestion, or the conditions for the second enzyme were set after incubation with the first enzyme.

2.19 Ligation of DNA

DNA ligases catalyze the linking of DNA molecules, consuming NAD⁺ or ATP through formation of a phosphodiester bond between a free 5' phosphate group and a 3' hydroxyl group. A three- to five-times excess of DNA fragment was incubated overnight at 12°C in ligation buffer with 100-500 ng cleaved plasmid and 5 units T4-DNA ligase as well as 10 nmol ATP. Only ligations of cohesive ends were carried out.

Ligation buffer:

40 mM	Tris-HCl pH 7.8
10 mM	MgCl ₂
10 mM	DTT
0.5 mM	ATP

2.20 DNA sequencing

(Sanger et al., Proc. Natl. Acad. Sci. USA 74 (1977) 5463-5467) The sequencing of DNA was carried out according to the chain-break method. Double-stranded plasmid DNA serves as substrate. Double-stranded DNA can be synthesized from single-stranded DNA starting from an oligonucleotide primer in the presence of dNTPs through a DNA polymerase. If a small proportion of dideoxynucleotides (ddNTPs) is contained in the nucleotide mixture, this leads to a randomly distributed incorporation of the ddNTPs, as the DNA polymerase cannot distinguish between dNTPs and ddNTPs. The incorporation leads to a breaking of the double-stranded synthesis, because of the absence of the 3'-hydroxyl group of the ddNTPs and, as it takes place statistically distributed, to DNA single strands of different lengths. As the synthesis mixture is divided in four and only one of the four ddNTPs is present in each sample, base-specific chain breaks result in the respective mixtures. To mark the synthesized single strand, α -³⁵S-dATP is added to the reaction. After denaturing and separation of the DNA via polyacrylamide gel electrophoresis, the bands can be autoradiographically detected and the sequence of the DNA strand read off direct.

The sequencings were carried out with the T7-Sequencing-Kit (Pharmacia) according to the manufacturer's instructions. Either the M13-Universal-Primer (Pharmacia) or Primer M13r was used as oligonucleotide primer. 32 μ l (approx. 2 μ g) of purified double-stranded plasmid DNA were denatured with 8 μ l 2 M NaOH for 10 mins at RT. After the addition of 7 μ l 3 M sodium acetate, pH 4.8, 4 μ l H₂O and 120 μ l -20° ethanol, the DNA was precipitated for 20 mins at -70°C. The DNA was isolated by centrifuging (15 mins, 4°C), washed twice with

-20° cold 70% ethanol and dried in a vacuum centrifuge. The DNA sediment was then resuspended in 10 µl H₂O and after the addition of 2 µl annealing buffer and 2 µl primer solution (10 pmol in water) hybridized with the oligonucleotide primer at 65°C for 5 mins, 37°C for 10 mins and 5 mins at RT. Directly afterwards, for the purpose of primer elongation and radioactive marking, 3 µl labelling mix, 1 µl α-³⁵S-dATP (1000 Ci/mmol, 10 µCi/µl) and 2 µl T7 polymerase solution (diluted 1:5 with enzyme dilution buffer) were added and incubated for 5 mins at RT. In each case, 4.5 µl of this mixture were placed onto a *MicroSample Plate* (Greiner) preheated to 37°C in which 2.5 µl each of the four different dNTP/ddNTP mixes were present. After five minutes' incubation at 37°C, which served for further elongation and base-specific termination, the reactions were ended by the addition of 5 µl stop solution. Before application to the polyacrylamide gel, the samples were denatured for 2 mins at 80°C. The samples were stored at -20°C.

Annealing buffer:

1 M	Tris-HCl pH 7.6
100 mM	MgCl ₂
160 mM	DTT

Labelling mix:

1.375 µM	dCTP
1.375 µM	dGTP
1.375 µM	dTTP
333.5 mM	NaCl

Enzyme dilution buffer:

20 mM	Tris-HCl pH 7.5
5 mM	DTT
100 µg/ml	BSA
5%	glycerine

Stop solution:

10 mM	EDTA
97.5%	formamide
0.3%	bromphenol blue
0.3%	xylene cyanol

A-mix:	840 µM dCTP	C-Mix:	840 µM dATP
	840 µM dGTP		840 µM dGTP
	840 µM dTTP		840 µM dTTP
	93.5 µM dATP		93.5 µM dCTP
	14 µM ddATP		14 µM ddCTP
	40 mM Tris-HCl pH 7.6		40 mM Tris-HCl pH 7.6
	50 mM NaCl		50 mM NaCl
G-Mix:	840 µM dATP	T-Mix:	840 µM dATP
	840 µM dCTP		840 µM dCTP
	840 µM dTTP		840 µM dGTP
	93.5 µM dGTP		93.5 µM dTTP
	14 µM ddGTP		14 µM ddTTP
	40 mM Tris-HCl pH 7.6		40 mM Tris-HCl pH 7.6
	50 mM NaCl		50 mM NaCl

2.21 Preparation of double-stranded DNA using oligonucleotides

The oligonucleotides were hybridized at 60°C for the preparation of the DNA mixture. 100 pmol were used per

nucleotide. 1 pmol of the hybridized sample was used for the PCR or 100 pmol for the Klenow reaction.

The following cycles were carried out for the PCR:

At the start:	10 min	94°C
30 cycles:	1 min	94°C
	1 min	45°C
	1 min	72°C
At the end:	10 min	72°C

1 pmol hybridized oligonucleotide was used as DNA *template* for the PCR. PCR primers were used for all PCR reactions carried out in a final concentration of 0.1 pmol/μl. 1 unit of the Taq polymerase was used per 50 μl of the reaction mixture. The concentration of the dNTPs in the PCR mixture was set at 0.1 mM.

To prepare the DNA mixture with the help of the Klenow reaction, 100 pmol of the hybridized oligonucleotides was dissolved in Klenow buffer (50 mM Tris-HCl, pH 8.0; 50 mM MgCl₂, 10 mM DTT, 0.05 mM dNTP). The reaction was started by the addition of 5 units DNA polymerase I (Klenow fragment). After 30 min at 37°C, the reaction was stopped by heating to 75°C (10 min).

2.22 Transfection of COS cells and CHO cells

5-10 μg linearized plasmid DNA are dissolved in 150 μl cell culture medium (without FKS and antibiotics). 30 μl of the transfection agent (SuperFect, Qiagen, Hilden) are added to the solution. The mixture is incubated for 10 min at RT and added to the cells after the addition of 1 ml medium. The

COS cells (80% confluent growth) were cultivated in 60 mm plates and washed with PBS shortly before the transfection. After an incubation of 3 h at 37°C and 5% CO₂, the transfection agent was removed. The cells were then washed 4 times with PBS and taken up in selection medium (DMEM, 5% FKS, 600 µg/ml Geneticin).

2.23 Chromatographic purification of the GP120 mixture

The chromatographic purification takes place according to standard methods (Techniques in HIV Research, Aldovini & Walker, Stockton Press, 1990; S.W. Pyle et al. Purification of 120,00 dalton envelope glycoprotein from culture fluids of human immunodeficiency virus (HIV)-infected H9 cells. AIDS Res Hum Retroviruses 1987, 3:387-400). Cell extracts and cell culture overhangs of GP120-expressing COS cells are used for the isolation of the GP120 mixture. After centrifuging (25,000 g), the lysate is purified as follows:

1. Gel filtration (Sephadex G-20)
2. Immunoaffinity chromatography (anti-GP120 antibodies bound to CH-sepharose 4B)
3. Concentration through protein precipitation
4. Concentration through dialysis

2.24 Preparation of the DNA vaccine

Starting material for the preparation of the DNA vaccine is the mixture of the BstEII-BamHI DNA fragments of the env gene which were used for the preparation of the GP120 protein mixture. A eukaryotic expression vector for the HIV-1 GP120 which is approved for DNA vaccinations (cf.

1. The first step in the process is to identify the problem or issue that needs to be addressed. This involves gathering information and understanding the context of the problem.

2. Once the problem is identified, the next step is to define the objectives and goals of the project. This helps to clarify what needs to be achieved and provides a clear direction for the team.

3. The third step is to develop a plan or strategy to address the problem. This involves breaking down the problem into smaller, manageable tasks and determining the resources needed to complete each task.

4. The fourth step is to implement the plan. This involves putting the strategy into action and monitoring progress regularly to ensure that the project is on track.

5. The final step is to evaluate the results of the project. This involves assessing the outcomes against the objectives and goals and identifying any areas for improvement.

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Patent Claims

1. Protein vaccine which comprises a mixture of viral protein molecules, **characterized in that** the molecules are sequence variants of a single viral protein or of part of same, the mixture containing $\geq 10^2$ sequence variants, which is obtainable by expression of a plasmid-DNA mixture which, because of the variation of nucleotide positions, contains randomly distributed sequence combinations.
2. Protein vaccine according to claim 1, **characterized in that** the mixture contains $\geq 10^3$ and preferably $\geq 10^4$ sequence variants.
3. Protein vaccine according to claim 1 or 2, **characterized in that** it comprises a mixture of GP120 proteins of HIV which in each case differ from each other in their amino acid sequence in the region of the V2 loop and/or of the V3 loop.
4. DNA vaccine which codes for a mixture of structurally different virus proteins, **characterized in that** the vaccine contains a mixture of sequence variants of a viral DNA molecule or of part of same, the mixture containing $\geq 10^2$ DNA molecules which differ from each other in their nucleic acid sequence, where the mixture, because of the variation of nucleotide positions, contains randomly distributed sequence combinations.

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5. DNA vaccine according to claim 4, **characterized in that** it contains a mixture of DNA molecules which code for the sequence variants of a viral protein or a part.
6. DNA vaccine according to claim 4 or 5, **characterized in that** the mixture contains $\geq 10^3$ and preferably $\geq 10^4$ DNA molecules which differ from each other in their nucleic acid sequence.
7. DNA vaccine according to one of claims 4 to 6, **characterized in that** it codes for a mixture of structurally different GP120 proteins of HIV, in which the vaccine contains a mixture of DNA molecules, the nucleic acid sequences of which differ from each other in the region coding for the V2 loop and/or in the region coding for the V3 loop.
8. DNA vaccine according to claim 7, **characterized in that** it contains a mixture of DNA molecules which differ from each other in their nucleic acid sequence such that they code for a mixture of GP120 proteins which contain amino acid sequences which differ from each other in the V2 loop and/or in the V3 loop.
9. Nucleic acid sequence which is derived from the env sequence represented in SEQ ID NO: 1 or a fragment of same, **characterized in that** it is modified such that it contains monovalent restriction sites which make possible the specific exchange of the V2 and V3 regions.

10. Nucleic acid sequence which is derived from the env sequence represented in SEQ ID NO: 1 or a fragment of same, **characterized in that** it is modified such that it contains ten monovalent restriction cleavage sites at intervals of approx. 150 base pairs.
11. Nucleic acid sequence according to claim 9 or 10, **characterized in that** the sequence is modified by the introduction of silent mutations.
12. Nucleic acid sequence according to claims 9 to 11, **characterized in that** it contains the sequence given in SEQ ID NO: 9.
13. Nucleic acid sequence, **characterized in that** it contains the sequence given in SEQ ID NO: 11.
14. Nucleic acid sequence, **characterized in that** it contains the sequence given in SEQ ID NO: 12.
15. Single-stranded nucleic acid sequence which contains the region coding for the V3 loop and/or for the V2 loop of GP120, **characterized in that** in the V3 loop a 247 bp-long BglIII-XbaI fragment or a 283 bp-long BglIII-NheI fragment is exchanged for a modified fragment, and in the V2 loop a 139 bp-long PstI-BclI fragment or a 339 bp-long PstI-EcoRI fragment is exchanged for a modified fragment, the fragment/the fragments in each case containing inosine, a nucleic acid exchange or a mutation at at least 6 positions.

16. Single-stranded nucleic acid sequence which contains the region coding for the V2 loop and/or the region of GP120 coding for the V3 loop, **characterized in that** in the V3 loop a 247 bp-long BglIII-XbaI fragment or a 283 bp-long BglIII-NheI fragment is exchanged for a modified fragment, and in the V2 loop a 139 bp-long PstI-BclI fragment or a 339 bp-long PstI-EcoRI fragment is exchanged for a modified fragment, the fragment/the fragments in each case containing inosine, a nucleic acid exchange or a mutation at at least 6 positions, the nucleic acid sequence being complementary to the nucleic acid sequence according to claim 14.
17. Nucleic acid sequence according to claim 15 or 16, characterized in that the fragment/the fragments contain(s) inosine, a nucleic acid exchange or a mutation at 9 to 20 positions.
18. Double-stranded DNA which comprises hybrids of the single-stranded nucleic acid sequence according to claim 15 or 17 with the single-stranded nucleic acid sequence according to claim 16 or 17.
19. Nucleic acid mixture which comprises double-stranded DNAs, the nucleic acid sequences of which are derived from the env sequence in SEQ ID NO: 1 or SEQ ID NO: 11 or a fragment of same, **characterized in that** the nucleic acid sequences in each case differ from each other in the region coding for the V2 loop and/or in the region coding for the V3 loop.

20. Nucleic acid mixture according to claim 19,
characterized in that the nucleic acid sequences
differ from each other such that they code for a
mixture of proteins which in each case contain amino
acid sequences which differ from each other in the V2
loop and/or in the V3 loop.
21. Nucleic acid mixture according to claim 20,
characterized in that the mixture contains $\geq 10^2$
sequence variants.
22. Nucleic acid sequence according to claim 21,
characterized in that the mixture contains $\geq 10^3$ and
preferably $\geq 10^4$ sequence variants.
23. Protein mixture which comprises sequence variants of
the GP120 protein, **characterized in that** it is a
mixture of proteins which contain amino acid sequences
which in each case differ from each other in the V2
loop and/or in the V3 loop, the mixture being
obtainable by expression of a plasmid DNA mixture
which, because of the variation of nucleotide
positions, contains randomly distributed sequence
combinations.
24. Protein mixture according to claim 23, **characterized**
in that the mixture contains $\geq 10^2$ sequence variants.
25. Protein mixture according to claim 24, **characterized**
in that the mixture contains $\geq 10^3$ and preferably $\geq 10^4$
sequence variants.

26. Plasmid, which contains an inserted double-stranded DNA according to claim 18.
27. Expression vector, **characterized in that** it contains an inserted nucleic acid sequence according to claims 9 to 14.
28. Expression vector according to claim 27, **characterized in that** it contains the sequence given in SEQ ID NO: 10.
29. Expression vector, **characterized in that** it corresponds to DSM 12612.
30. Vector mixture which contains a mixture of plasmids according to claim 26, **characterized in that** the nucleic acid sequences of the plasmids differ in each case from each other in the region coding for the V2 loop and/or in the region coding for the V3 loop, where the mixture of the plasmids, because of the variation of nucleotide positions, contains randomly distributed sequence combinations.
31. Vector mixture according to claim 30, **characterized in that** the mixture contains $\geq 10^2$ plasmids which differ from each other in their nucleic acid sequence.
32. Vector mixture according to claim 31, **characterized in that** the mixture contains $\geq 10^3$ and preferably $\geq 10^4$

plasmids which differ from each other in their nucleic acid sequence.

33. Vector mixture according to one of claims 30 to 32, **characterized in that** the plasmids can be expressed in *E. coli* as host cell.
34. Vector mixture according to one of claims 30 to 32, **characterized in that** the plasmids can be expressed in eukaryotic cells, preferably in Cos, CHO or BHK cells, as host cells.
35. *E. coli* host cells which are transfected with a vector mixture according to claim 33.
36. Eukaryotic host cells which are transfected with a vector mixture according to claim 34.
37. Eukaryotic host cell according to claim 36, **characterized in that** they are a host cell from the group consisting of Cos, BHK or CHO cells.
38. Process for the preparation of the nucleic acid sequence according to claim 10, **characterized in that** so many silent mutations are introduced into a nucleic acid sequence coding for a viral protein that the nucleic acid sequence thereby obtained contains monovalent restriction sites which make possible the exchange of the V2 and V3 regions.
39. Process for the preparation of the nucleic acid sequence according to claim 10, **characterized in that**

so many silent mutations are introduced into a nucleic acid sequence coding for a viral protein that the nucleic acid sequence thereby obtained contains ten monovalent restriction sites at intervals of approx. 150 base pairs.

40. Process according to claim 38 or 39, **characterized in that** the nucleic acid sequence coding for a viral protein is the sequence according SEQ ID NO: 1 or SEQ ID NO: 11 or a fragment of same.
41. Process for the preparation of the vector mixture according to claims 33 and 34, **characterized in that** plasmids, the nucleic acid sequences of which in each case differ from each other in the region coding for the V2 loop and/or in the region coding for the V3 loop in each case through random distribution of the bases at the varied nucleotide positions, are ligated into a vector which can be expressed in host cells.
42. Process according to claim 41, characterized in that the host cells are *E. coli*, Cos, CHO or BHK cells.
43. Process for the preparation of the host cells according to claim 35 or 36, **characterized in that** the host cells are transformed with a vector mixture according to claims 30 to 32.
44. Process for the preparation of a protein vaccine according to one of claims 1 to 3, **characterized in that** the host cells are cultivated according to one of claims 35 to 37 under conditions which allow the

expression of the mixture of viral protein sequence variants.

45. Process for the preparation of a DNA vaccine according to one of claims 4 to 8, **characterized in that** the process is carried out according to claim 41 or 42, the plasmids according to the invention being ligated into a vector which can be expressed in host cells of the organism to be vaccinated.
46. Use of a mixture of structurally different viral proteins which are sequence variants of a viral protein or of part of same, for the preparation of a vaccine for the prevention and/or therapy of a virus infection in humans.
47. Use of a protein mixture according to one of claims 23 to 25 for the preparation of a vaccine for the prevention and/or therapy of a HIV infection in humans.
48. Use of a mixture of DNA molecules which code for sequence variants of a viral protein or of part of same, for the preparation of a vaccine for the prevention and/or therapy of a virus infection in humans.
49. Use of a nucleic acid mixture according to claims 19 to 22 for the preparation of a vaccine for the prevention and/or therapy of a virus infection in humans.

50. Use of the nucleic acid mixture according to one of claims 19 to 22 for the preparation of a vector mixture according to one of claims 30 to 32 which can be expressed in host cells , the host cells being selected from the group consisting of *E. coli*, Cos, CHO and BHK cells.
51. Use of the vector mixture according to one of claims 30 to 32 for the expression of a protein mixture according to one of claims 23 to 25.
52. Use of the host cell according to one of claims 35 to 37 for the preparation of a protein mixture according to one of claims 23 to 25.
53. Pharmaceutical composition for the prevention and/or therapy of a virus infection, **characterized in that** it comprises a protein mixture and a nucleic acid mixture, the protein mixture comprising sequence variants of a viral protein or of part of same, and the nucleic acid mixture comprising DNA molecules which code for sequence variants of a viral protein or of part of same.
54. Pharmaceutical composition according to claim 53, **characterized in that** it comprises a protein mixture according to claims 23 to 25 and a nucleic acid mixture according to claims 19 to 22.

Abstract

The present invention relates to a pharmaceutical composition or a vaccine which comprises a mixture of viral protein molecules which are sequence variants of a single viral protein or of part of same. The invention furthermore relates inter alia to a DNA vaccine which codes for a mixture of structurally different virus proteins, the vaccine containing a mixture of sequence variants of a viral DNA molecule or of part of same which code for sequence variants of a viral protein or part. According to a preferred version of the invention, the viral proteins are sequence variants of the GP120 protein of the human immunodeficiency virus (HIV) which differ from each another in their amino acid sequence in the region of the V2 loop and/or of the V3 loop. The invention furthermore relates to the preparation of the virus vaccines including the intermediate stages or constructs, preparation processes and uses connected with them.

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Fig. 1

V3 loop sequence data of HIV-1 patient isolates (PI)

Fig. 2a

Amino acid sequences of the NL4-3 V2 loop and NL4-3 V3 loop. The regions which are preferably to be varied are underlined.

Fig. 2b

Variation of the V3 loop. Data from the Los Alamos database.

Fig. 4

Schematic representation of the process for the preparation of the mixture of gp 120-expressing plasmid vectors.

- 1.) Preparation of the degenerated DNA fragments for e.g. the V3 loop
 - a.) Synthesis of single-stranded DNA
 - b.) Hybridization of two complementary oligonucleotides.
- 2.) Cloning of the V3 loop DNA fragments into pUC18 delta env
- 3.) Cloning of the env gene into the gp 120 expression vector pBSCenvATG

V3 loop fragments with degenerated sequence e.g.:

	1970	1971	1972	1973	1974	1975	1976	1977	1978	1979	1980	1981	1982	1983	1984	1985	1986	1987	1988	1989	1990	1991	1992	1993	1994	1995	1996	1997	1998	1999	2000	2001	2002	2003	2004	2005	2006	2007	2008	2009	2010	2011	2012	2013	2014	2015	2016	2017	2018	2019	2020	2021	2022	2023	2024	2025	2026	2027	2028	2029	2030	2031	2032	2033	2034	2035	2036	2037	2038	2039	2040	2041	2042	2043	2044	2045	2046	2047	2048	2049	2050	2051	2052	2053	2054	2055	2056	2057	2058	2059	2060	2061	2062	2063	2064	2065	2066	2067	2068	2069	2070	2071	2072	2073	2074	2075	2076	2077	2078	2079	2080	2081	2082	2083	2084	2085	2086	2087	2088	2089	2090	2091	2092	2093	2094	2095	2096	2097	2098	2099	2100	2101	2102	2103	2104	2105	2106	2107	2108	2109	2110	2111	2112	2113	2114	2115	2116	2117	2118	2119	2120	2121	2122	2123	2124	2125	2126	2127	2128	2129	2130	2131	2132	2133	2134	2135	2136	2137	2138	2139	2140	2141	2142	2143	2144	2145	2146	2147	2148	2149	2150	2151	2152	2153	2154	2155	2156	2157	2158	2159	2160	2161	2162	2163	2164	2165	2166	2167	2168	2169	2170	2171	2172	2173	2174	2175	2176	2177	2178	2179	2180	2181	2182	2183	2184	2185	2186	2187	2188	2189	2190	2191	2192	2193	2194	2195	2196	2197	2198	2199	2200	2201	2202	2203	2204	2205	2206	2207	2208	2209	2210	2211	2212	2213	2214	2215	2216	2217	2218	2219	2220	2221	2222	2223	2224	2225	2226	2227	2228	2229	2230	2231	2232	2233	2234	2235	2236	2237	2238	2239	2240	2241	2242	2243	2244	2245	2246	2247	2248	2249	2250	2251	2252	2253	2254	2255	2256	2257	2258	2259	2260	2261	2262	2263	2264	2265	2266	2267	2268	2269	2270	2271	2272	2273	2274	2275	2276	2277	2278	2279	2280	2281	2282	2283	2284	2285	2286	2287	2288	2289	2290	2291	2292	2293	2294	2295	2296	2297	2298	2299	2300	2301	2302	2303	2304	2305	2306	2307	2308	2309	2310	2311	2312	2313	2314	2315	2316	2317	2318	2319	2320	2321	2322	2323	2324	2325	2326	2327	2328	2329	2330	2331	2332	2333	2334	2335	2336	2337	2338	2339	2340	2341	2342	2343	2344	2345	2346	2347	2348	2349	2350	2351	2352	2353	2354	2355	2356	2357	2358	2359	2360	2361	2362	2363	2364	2365	2366	2367	2368	2369	2370	2371	2372	2373	2374	2375	2376	2377	2378	2379	2380	2381	2382	2383	2384	2385	2386	2387	2388	2389	2390	2391	2392	2393	2394	2395	2396	2397	2398	2399	2400	2401	2402	2403	2404	2405	2406	2407	2408	2409	2410	2411	2412	2413	2414	2415	2416	2417	2418	2419	2420	2421	2422	2
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V3 loop sequence:

Degeneration at protein level:

variants

variants

Variability at protein level derived from the degenerated DNA sequence:

As a below named inventor, I hereby declare that my residence, mailing address and citizenship are as stated below next to my name, and I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

the specification of which (check applicable box(s)).

☐ is attached hereto
☐ was filed on _____ as U.S. Application Serial No. _____ (Atty Dkt. No. 35-213)
☒ was filed as PCT International application No. _____ PCT/EP99/09759 on 3 December 1999
 and (if applicable to U.S. or PCT application) was amended on 2 November 2000

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above. I acknowledge the duty to disclose to the Patent Office all information known to me to be material to patentability as defined in 37 C.F.R. 1.56. I hereby claim foreign priority benefits under 35 U.S.C. 119/365 of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed or, if no priority is claimed, before the filing date of this application:

Priority Foreign Application(s):

Application Number	Country	Day/Month/Year Filed
199 07 485.2	GERMANY	12 February 1999

I hereby claim the benefit under 35 U.S.C. §119(e) of any United States provisional application(s) listed below.

Application Number Date/Month/Year Filed

I hereby claim the benefit under 35 U.S.C. 120/365 of all prior United States and PCT international applications listed above or below.

Prior U.S./PCT Application(s):

Application Serial No. PCT/EP99/09759

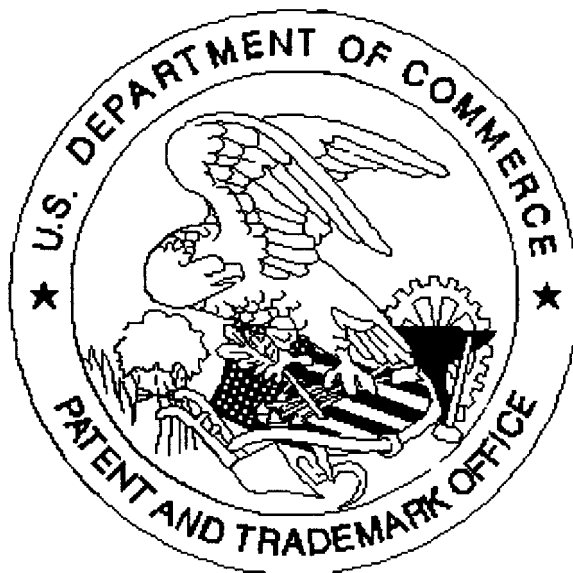
Day/Month/Year Filed 3 December 1999

Status: patented
pending, abandoned

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon. And on behalf of the owner(s) hereof, I hereby appoint **NIXON & VANDERHYE P.C., 1100 North Glebe Rd., 8th Floor, Arlington, VA 22201-4714, telephone number (703) 816-4000 (to whom all communications are to be directed)**, and the following attorneys thereof (of the same address) individually and collectively owner's/owners' attorneys to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith and with the resulting patent: Larry S. Nixon, 25640; Arthur R. Crawford, 25327; James T. Hesmer, 30184; Robert W. Faris, 31352; Richard G. Besha, 22770; Mark E. Nusbaum, 32348; Michael J. Keenan, 32106; Bryan H. Davidson, 30251; Stanley C. Spooner, 27393; Leonard C. Mitchard, 29009; Duane M. Byers, 33363; Jeffrey H. Nelson, 30481; John R. Lastova, 33149; H. Warren Burnam, Jr., 29366; Mary J. Wilson, 32955; J. Scott Davidson, 33489; Alan M. Kagen, 36178; Robert A. Molan, 29834; B. J. Sadoff, 36663; James D. Berquist, 34776; Updeep S. Gill, 37334; Michael J. Shea, 34725; Donald L. Jackson, 41090; Michelle N. Lester, 32331; Frank P. Presta, 19828; Joseph S. Presta, 35329; Joseph A. Rhoa, 37515; Raymond Y. Mah, 41426; Chris Comuntzis, 31097. I also authorize Nixon & Vanderhye to delete any attorney names/numbers no longer with the firm and to act and rely solely on instructions directly communicated from the person, assignee, attorney, firm, or other organization sending instructions to Nixon & Vanderhye on behalf of the owner(s).

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